TRANSMISSIBLE DRUG RESISTANCE, PLASMID CHARACTERIZATION IN SHIGELLA AND SALMONELLA, AND VIRULENCE OF SHIGELLA ISOLATED FROM DIARRHOEIC HUMANS AND PIGLETS

BY

ISAAC ADEYEMI ADELEYE

B.Sc. (Hons.) Lagos,
M.Sc. (Microbiology) IFE.

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ABSTRACT

A total of 10,200 faecal samples including 10,000 from diarrhoeic human beings and 200 from diarrhoeic piglets were collected. The human faecal samples were collected from the three government hospitals in Ibadan, namely: The University College Hospital, State Hospitals at Adeoyo and Ring Road, while the piglets were on the Teaching and Research Farm, of the University of Ibadan.

Thirty Shigella and twenty-two Salmonella spp. were isolated from the human faecal samples while one Shigella isolate was obtained from the faecal samples of piglets. The Shigellae were serologically identified as S. flexneri (23 from humans and 1 from a piglet), S. dysenteriae (4 isolates from human) and S.boydii (3, all were isolated from human). Of the Salmonella isolates, 9 were identified as S.typhi while the remaining 13 were classified as Salmonella species. One microgram per ml potassium tellurite in MacConkey agar was used to enhance the isolation of Shigellae.

Antimicrobial susceptibility testing to eight antibiotics were peformed on the fifty -three bacterial isolates. Twenty-one antibiotic resistance patterns were identified.

The highest pattern T-CT-F-A-S-C-Te (Septemdriple) was found in eleven isolates, sextiple resistant pattern in thirteen; quintiple pattern in eight isolates; quadriple in four isolates; triple in eight isolates; double in four isolates while resistance to single antibiotic (Te and CT) was found in five bacterial isolates.

The Minimal Inhibitory Concentrations (MIC) of 4 antibiotics (ampicillin, chloramphenicol, streptomycin and tetracycline) were determined for the bacterial isolates. Two of the Shigella isolates were sensitive to ampicillin, the MIC of ampicillin was 8 ug per ml for them, 1 to chloramphenicol; 4 to streptomycin while all isolates were resistant to tetracycline. The MIC of ampicillin for 14 of the Salmonella isolates was 8 ug per ml while 11 and 9 isolates of the same organism were resistant to chloramphenicol and streptomycin respectively. The following reistance patterns were observed: A-C-S-T, C-S-T, A-C-T and A-S-T.

All the forty-two isolates screened transferred ampicillin resistance (100 percent). Twenty-one isolates (50.0 percent) transfered two determinants, either A-T or A-S or A-C. Seven (16.6 percent) transfered three determinants either A-T-S or A-S-C.

None of the isolates transfered four determinants (A-T-S-C). Among the Shigella isolates the chloramphenicol determinant was transfered at a low frequency (only 3 of the 28 screened) whereas the Salmonella isolates transfered at a higher frequency (6 of the 14 screened).

The R plasmids range in size between 2.2 to 38.0 Mdal. The chloramphenicol R plasmid has a molecular weight of 4.00 Mdal., streptomycin 36.0 Mdal. and tetracyline 38.0 Mdal.

The plasmid profile of the isolates was also investigated using agarose gel electrophoresis method. The Shigella isolates exhibited a large number of small cryptic plasmids. In contrast, the Salmonella isolates exhibited fewer number of plasmids. All the plasmids range between 0.8 and 55.5 Mdal. in size.

The virulence of the Shigella isolates was investigated using Sereny Test and Rabbit ligated ileal loop test. Four of the fourteen Shigella isolates including S.flexneri (2), S. boydii (1), S.dysenteriae (1) produced Keraconjuctivities in guinea pigs.

Six of the Shigella isolates including four S.flexneri, one S.dysenteriae and one S.boydii dilated ligated rabbit ileal loop with accumulation of fluid. Histological ulterations found in the ileal loops exposed to these

enterotoxins included inflamation, general degeneration, submucosal oedema and neutrophilic infiltrations.

Four of the Shigella isolates comprising of two S. flexneri, one S. dysenteriae and one S. boydii dilated the ligated intestinal loop of rabbit with accumulation of fluid when enterotoxin heated at 65 C for 30 min. were used. It was observed for the first time that the S.boydii produced heat labile and heat stable enterotoxin in ligated rabbit illeal loop.

Oral inoculation of the invasive Shigella isolates into pretreated (starved and calcium carbonate treated) mice and guinea pigs failed to produce clinical manifestation of dysentery-like diarrhoea and febrile condition.

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DEDICATION

To (i) The Adeleyes' for making me what I am.

(ii) Josephine for her love and understanding.

CERTIFICATION

I certify that this work was done by Isaac Adeyemi Adeleye in the Department of Veterinary Microbiology and Parasitology University of Ibadan.

DR. A. I. ADETOSOYE
SUPERVISOR.

DVM (ABU), Dr. Med. Vet. (VIENNA),
Cert. in Animal Management &
Reproductive Biology (VIENNA)
Ph.D. (IBADAN).

PROF. M. OLA. OJO,
SUPERVISOR.

KSG, BVM&S, MRCVS, (EDIN.),
Dip. Bact. (LOND.),
Ph.D. (IBADAN).

TABLE OF CONTENTS

CHAPTER 2

CHAPTER 6

LIST OF TABLES

FIGURE PAGE

CHAPTER ONE

INTRODUCTION

Infectious enteric diseases, including acute diarrhoeal disease are of great public health importance in developing and underdeveloped countries of the world.

Diarrhoea has long been recognised as the greatest killer of infants and young children in the developing countries (WHO Scientific Working Group, 1983). Well over five hundred million episodes of diarrhoea in children under five are estimated to occur annually in Asia, Africa and Latin America with about five million deaths recorded in 1980 (Cutting and Elliot, 1980). Diarrhoea is often lethal among the very young. It is also a major cause of ill-health and death among children and adult of all ages when badly managed.

In the investigations of bacterial etiology of diarrhoeal infections, Shigella has been recognised as a pathogen of man and primates (Tiwary and Prasad, 1972). However, S. flexneri, S. sonnei and S. boydii have been isolated from diarrhoea and faecal materials of dogs (Doem and Mortelmans, 1954; Floyd, 1955 and Carwadine, 1982).

Nyaga et al. (1985) reported the isolation of S. dysenteriae 3 organism, a human pathogen, from poultry in Kenya. Ibu et al. (1987) also reported a case of fatal Shigellosis caused by S. flexneri in piglets in Jos (Nigeria).

Many enteric gram negative organisms e.g. Salmonella spp and some strains of Campylobacter infect man as well as animals (Coker and Dosunmu-Ogunbi, 1984). The larger proportions of Salmonella infections in man are derived from eating contaminated meat and therefore, indirectly from animal sources. Domesticated animals like dogs and cats have been known to be carriers of Salmonella (Khan, 1970) while frogs, lizard and snakes are actual reservois of this organism (Sarvamangala and Shivanda, 1983). Other wellknown causes of diarrhoea are V. cholerae and certain strains of E.coli.

In the last ten years owing to active research in different parts of the world, various agents including rotavirus, Campylobacter, Yersinia, Aeromonas, P. aeruginosa Plesiomonas, C. perfiring es have been associated with diarrhoea (Candy, 1984; Reunels et al., 1985 and Williams et al., 1985).

Rotavirus which was first reported in children in 1973 is known to cause perhaps fifty percent of childhood diarrhoea, the incidence increases to eighty percent in temperate climates (Banatvala, 1979; Malta et al., 1983

In addition to bacteria and viruses, parasites have also been implicated in the outbreak of diarrhoea. The most important protozoan parasities known to cause diarrhoea are Giardia lamblia, Entamoeba histolytica and Cryptosporidium (Rhode, 1984). Among helminths, Strongyloide and occasionally, Trichuris have been known to be causative agents of diarrhoea. The mechanism of amoebic dysentery is related to adherence through microfilament invasion and microabscess formation (Radvin and Guerrant, 1981).

Shigellosis (Bacillary dysentery) has attracted attention throughout the course of history by its dramatic clinical manifestations. It was described in the Bible in association with rectal proplase ("Emrods"). Hippocrates described it as the bloody flux and Thomas Willis as a "dysentery" in the course of a "putrid fever" (Schwartz and Bishop 1958). It has been associated with major mobidity and often high mortality wherever it has struck.

The Shigellae are thin, non-motile, non-sporulating gram negative rods closely related to Escherichia coli in the family Enterobacteriaceae. There are four related Shigella species namely S. dysenteriae, S. flexneri, S. boydii

and S. sonnei differentiated by biochemical and serological reactions. Within each species a number of distinct subspecies are grouped together, all sharing major O (somatic) antigenic determinants, classified as A,B,C and group D respectively. They all ferment glucose but do not produce gas (except for a few isolates of S. flexneri) and they neither ferment lactose (except S. Sonnei which turns positive after sometime in broth culture) nor produce H_2S (Burrows et al., 1968).

These few properties are the basis of microbiological diagnosis of the infection, which relies upon differential inhibitory agar media to suppress growth of gram-positive organisms and coliforms and to reveal the capacity of the latter to rapidly ferment lactose.

Shigella dysenteriae is the classical agent of invasive diarrhoea. The organism elaborates a variety of toxins (neurotoxin, cytotoxin and exterotoxin) many of which are plasmid encoded, and may facilitate penetration of the mucosa of the colon (Keusch, 1979). Shigella dysenteriae is cytotoxic and moves laterally to invade contiguous cells. Microabscesses and denuded ulcers lead to bloody dysentery.

Salmonellosis is one of the zoonotic diseases of public health importance. The natural habitat of members of the genus Salmonella is the intestinal tract of warm-blooded and many cold-blooded vertebrates from where they may survive and even multiply.

In general, members of the genus can be divided into three categories on the basis of their host predeliation:

1. Man adapted Salmonellae:

Those primarily adapted to man i.e. the enteric fever organisms, S. typhi and S.paratyphi A,B,C belong to this category. S.typhi and S.paratyphi A and C have no known secondary hosts and hence are rarely isolated from animals other than man. S. paratyphi B, while primarily adapted to man, is occasionally isolated from seondary hosts including cattle, swine, dogs and fowls (USDA Committee on Salmonella, 1969; Anon, 1971).

2. Animal adapted Salmonellae:

Those primarily adapted to particular animal hosts, for example, S. cholera-suis (pigs); S. dublin cattle; S. pullorum and S. gallinarium (chickens); S. arbortus-equi (horses) and S. abortus-ovis (sheep).

These are all capapble of causing clinical illness in man, although only S. cholera-suis and S. dublin are of any significant consequence in this context.

3. Unadapted Salmonellae:

The remainder of the more than two thousand serotypes of the genus have no apparent host preference and belong to this category. They are all potential agents of human infection, but, in practice, the real problem of human Salmonellosis is limited to a relative handful serotypes (CDC Salmonella Survillance, 1976).

The epidemiology of Salmonellae of categories 2 and 3 with respect to infection in man mostly involves the animal reservoir and transmission through food in which multiplication may take place. The most common clinical manifestation of Salmonella infection is gastro-enterocolitis. After incubation period of 6 to 48 hours (usually 12 to 24 hours) headache, malaise, nausea, vomitting and diarrhoea appear abruptly. Abdominal pain is frequent and may cause mild to severe discomfort. The patient may have fever and shivering (Yoshikawa et al., 1980). In complicated cases, the acute stage usually terminates within forty-eight hours, although it may be

prolonged and the patient may not feel well for several more days. The diarrhoea may also be severe with frequent passage of watery, greenish, offensive stools with mucus; blood may also be present. Abdominal cramps and diarrhoea may persist after nauseaa and vomitting have ceased (Mandal, 1979).

Diarrhoea, whatever the etiology, leads to acute fluid and electrolyte loss (Rohde and Northrup, 1976). This loss of fluid and electrolytes leads to dehydration and, in some cases death if not quickly arrested.

In the treatment of diarrhoea the following steps are usually considered (Mandal, 1979):

(i) Prevention of contact with the infecting agent;

(ii) replacement of sodium, bicarbonate, potassium, substrate and water loss;

(iii) interference with the mechanism of diarrhoea;

(iv) prevention of infection through the use of vaccine; and

(v) use of antibiotics.

(1) <u>Prevention of contact with the infecting agent:</u>

Bacterial diarrhoea (Shigellosis and Salmonellosis) is known to spread through contact

with faeces, food, foul water and by "fingers" (Blaser and Newman, 1982). Thus interruption of the faecal-oral transmission of diarrhoea is accomplished through widespread introduction of clean water supplies, personal and food hygiene and sanitary waste disposal (Rhode, 1984).

Inadequate refrigeration of stored foods encourages growth of organisms causing acute food poisoning. Salmonella organisms are spread through the mass processing of poultry. Control of this and other factors is part of the complex health regulations of the food chain necessary to interrupt transmission of diarrhoea causing pathogens.

Replacement of sodium, bicarbonate, potassium substrate and water loss:

The development of oral fluid therapy for diarrhoea, which was the outcome of research on Vibrio cholerae demonstrated the high efficacy of replacement of water and electrolytes with an appropriate, absorbable substrate, thereby off-setting the effects of dehydration and substantially reducing the number of deaths. Extensive reviews have documented both scientific basis and the efficacy of oral rehydration therapy (ORT) in acute diarrhoea (Rohde, 1984; Candy, 1984). This is being extensively

practiced in Bangladesh as well as other third world countries including Nigeria. The principle involved is that of co-transport of sodium equimolar with active transport of sugar (Keusch, 1979).

3. Interference with the mechanism of Diarrhoea:

A number of antisecretory drugs (aspirin, indomethacin, and chlorpromazine) working through a variety of mechanisms in the mucosal cells have been shown to diminish or reverse the secretory process and, therefore, effectively reduce loss of fluid and electrolyte (Rhode, 1984). For example Aspirin and Indomethacin have been shown to stimulate Sodium and Chlorine absorption in normal tissue and it may be this activity which is responsible for their effect on cholera toxin (Turnberg, 1978). Chloropromazine prevents intestinal secretion in the mouse by inhibiting adenylcyclase activity (Lonroth et al., 1977).

4. Prevention through the use of vaccines:

For oral vaccines against Shigella, five doses and booster doses were required (Levine et al, 1976).

In the case of Salmonella, the Ty21a oral typhoid vaccine appears to offer some protection, even in single doses (Wahdan et al., 1982) but there are still no vaccines for the over two thousand or more serotypes of common Salmonella. Recombinant DNA technologies make production of a wide variety of specific antigens possible and open the way for eventual polyvalent vaccines aimed against a host of organisms. For example Shigella-Salmonella hybrid has been created (Formal et al., 1981) by transfering plasmids responsible for form 1 antigen synthesis in Shigella to established gal E. S. typhi strain. At present research is still going on in various laboratories in the world to find a suitable vaccine to combat the menace of Salmonella and Shigella infections.

Use of Antibiotics:

Several authors have advanced three congent reasons for reconsidering the use of anti-microbials in the therapy of Shigellosis (Weissman et al., 1973): the disease is usually self-limiting and may be mild (especially that due to S. sonnei); post-infection carriage is usually less than three to four weeks and with increasing frequency, the organism possesses plasmids

coding for transferable drug resistance (Anderson, 1968 and Weissman et al., 1974). In cases in which the invasive organism causes a dysentery-like syndrome, the specific drug of choice is based on knowledge of sensitivity patterns of the pathogen when cultured in the same environment.

In case of enteric fever caused by Salmonella typhi, chloramphenicol has been the most successful drug. The clinical response to the drug has been fairly uniform throughout the world (Mandal, 1979). A rapid improvement in the patient's general condition followed be defervescence within two to five days has been recognised. However, the wide-scale emergence of R-factor mediated resistance to chloramphenicol (as well as other antibiotics) in S. typhi (Datta, 1965) has introduced a new sense of urgency in the search for suitable antibiotics.

Problem of plasmid mediated tranmissible drug resistance:

Many of the antibiotics used in treating human infections are also used as chemotherapeutic and prophylactic agents in animals as well as feed additives at low concentrations to increase their growth rate and reduce economic losses of animals (Walton, 1966a; Ojo and Adetosoye, 1977).

Soon after the introduction of antibiotics into medical practice and as feed additives, antibiotics resistance amongst bacteria emerged as a significant problem.

Studies on the nature of antibiotic resistance in bacteria reveal that several biochemical mechanisms may be responsible. These include:

(a) alteration of target site in the cell that eliminate or decrease the binding of the drug;

(b) decreased drug accumulation into the cell; and

(c) antibiotic inactivation by enzymes, etc.

Resistance of type a, which arise by mutational alteration of a cellular component are very common in laboratory derived resistant strains but clinical isolates of this have also been reported (Benveniste and Davies 1973).

Resistance of types b and c are generally found only in clinical isolates of resistant bacteria, and are normally associated with extrachromosomal element or plasmid (O'Brien, 1987). The plasmids carry "resistance factors" or R-factors. Transfer is effected by contact resulting in conjugation between drug resistant donor cells and senstive recipient cells.

Apart from transmissible drug resistance, plasmids also play an important role in the pathogenicity of Shigella and Salmonella. Takabashi et al. (1988) demonstrated that large plasmid (150 megadalton) found in S. boydii was responsible for the epithelial cell penetration by the organism. There is also a correlation between the presence of a fifty megadalton plasmid in Salmonella dublin and its virulence for mice (Tarakado et al., 1983).

Central to the studies on Shigella and Salmonella is the question of the virulence. Levine et al. (1973) established that the primary virulence determinants of Shigellae is the invasive capacity of the organism as strains which cannot penetrate and multiply within the colonic epithelial cells do not cause disease in humans. It is also know that S. flexneri adhere very well to the colonic mucosa of guinea pig in the presence of adhesin (binding protein) found in the intestinal cells (Izhar et al., 1982).

Shigella dysenteriae is known to elaborate a toxin with cytotoxic, enterotoxic and neurotoxic properties. This heat labile toxin is enterotoxic when injected into rabbit illeal loops (Cavanagh et al., 1956; Keusch et al., 1972a) and lethal to mice and rabbits (Van Heyningen and Gladstone, 1953). Moreover, it is cytotoxic for

eukaryotic cells such as HeLa cells (Keusch et al., 1972b), KB (human epidermal carcinoma), normal human liver and monkey kidney (Vicari et al., 1960). Furthermore, purified Shigella toxin inhibits protein synthesis in cell-free translation system (Olenick and Wolfe, 1980).

Likewise cell-free extract of Salmonella has been found to inhibit protein synthesis and cause cytotoxicity in eukaryotic cells (Koo and Peterson, 1982).

Previous work on Salmonella and Shigella in Nigeria:

Salmonella spp. have been isolated from various animals in Nigeria: cattle (Collard and Sen, 1956). fowls (Sen and Collard, 1957a) pig (Sen and Collard,1957b,) agama lizard (Collard and Montefiore, 1956), goats (Falade, 1976) and captive animals (Falade and Durojaiye, 1977). Other workers, Oduye and Olayemi (1977) and Britt et al. (1978) have also reported the isolation of Salmonellae from dogs.

The isolation of Salmonella serotypes from man was first reported by Collard and Sen, (1957). The picture of Salmonellosis in Nigeria is one of wide diversity of types in both man and animals. Many of the Salmonella types obtained from man had also widespread distribution in other sources, whereas other types were limited to man only.

Perhaps at one end of the scale would be S. typhi which has not been isolated from other sources other than man and at the other end would be S. agama which has been found in several sources.

In contrast, the distribution of Shigella in Nigeria is less widespread. It has been isolated from man and a few domestic animals. Collard and Sen (1961) reported 691 strains of Shigella over a five-year period (1956-61). Of these 71.3% were S. flexneri, 6.1% S. boydii while S. sonnei was least common. Odugbemi et al. (1982) recorded a higher percentage of Shigella (3.9%) than Salmonella (0.9%) infections in children under five years of age in Lagos. The most common specie was found to be S. flexneri. Shigella has also been isolated from diarrhoeic piglets and fowls (Ibu et al., 1987).

Chemotherapeutics and Bacterial resistance in Nigeria:

Diarrhoea caused by Salmonella and Shigella is very common in Nigeria (Odugbemi et al., 1982). The drugs used in treating this condition include ampicillin, streptomycin, tetracycline and chloramphenicol (Adetosoye and Rotilu, 1986). The laws controlling the sales of these drugs are not enforced in Nigeria. As a result

antimicrobial agents are easily purchased by individuals without prescription; they are sold in motor parks and market places. Thus the indiscriminate use of these agents in Veterinary and human medicine has resulted into emergence of drug resistance (Ojo, 1973; Osoba, 1979; Ogunnariwo, 1987).

The first case of chloramphenicol resistant Salmonella in Nigeria was reported by Njoku-Obi and Njoku-Obi (1965). Ojo (1973) isolated twenty-four strains of Salmonella from animals in Ibadan and tested them for the sensitivity to eleven chemotherapeutic agents. Two of the isolates were found to show multiple resistance to chloramphenicol, sulphonamide, streptomycin, tetracycline, ampicillin and septrin. Ibe et al. (1987) isolated S. flexneri which were found to be resistant to septrin, ampicillin, penicillin, streptomycin, neomycin and erythromycin from diarrhoeic piglets.

Some of the resistant strains have been found to harbour R-factor. Adetosoye and Rotilu (1986) reported the presence of drug resistance plasmids in twenty-four S. typhimurium which were isolated from calves with diarrhoea and fifteen other Salmonella spp. as well as

twenty-four *Shigella* isolates which were isolated from diarrhoeric children in Ibadan.

From these studies it is apparent that drug resistance is the major cause of treatment failures in Nigeria.

OBJECTIVES OF STUDY

Diarrhoeal diseases of various causes including bacteria, virus and parasitic agents are common and of great public health importance in Nigeria. They are also of great economic importance to the livestock industry. There is some evidence that a proportion of this disease is caused by Shigella and Salmonella (Akinkugbe et al., 1968; Alonge and Oyekole, 1982; Odugbemi et al., 1982; Adetosoye and Rotilu, 1986).

This study is undertaken to:

(i) Isolate Salmonella and Shigella from diarrhoeic humans and piglets.

(ii) Characterise such isolates biochemically and serologically.

(iii) Determine whether there is transmissible drug resistance among the isolates.

(iv) Isolate and characterise the plasmids harboured by the isolates.

(v) Study the pathogenicity of Shigella isolates using various animal models.

CHAPTER TWO

LITERATURE REVIEW

The first dysentery bacillus was isolated and identified by Kiyoshi Shiga in Japan in 1898 during a severe bacillary dysentery epidemic. Morphologically, it was described as short, plump and aniline stainable bacillus. Kruse in 1900 isolated bacteria that resembled those of Shiga from dysentery patients in West Phalia. Flexner in the same year reported the isolation of microorganism he thought looked like Shiga's bacillus from American soldier in the Philipines. Strong and Mustgrave (1900) confirmed the work of Flexner and produced dysentery with the bacteria in a monkey and in a condemned criminal. Castellani (1904) described an acute case of bacillary dysentery which led to death. He found at autopsy that the mucosa of the colon and rectum were thickened, covered with mucus and studded with a large number of very small superficial roundish ulcers.

These reports were followed in rapid succession by a large number of publications from virtually all parts of the world which recorded the isolation of these and similar microogranisms from acute and chronic cases of dysentery

(Edward and Ewing, 1972). However, important advances in the knowledge of this disease was not made until the 1940s. This was largely through the work of Boyd (1940) who described a number of Shigella paradysenteriae from India; Sachs (1943) isolated non-mannitol-fermenting Shigella from Middle-East; Christensen and Gowen (1944) characterized two strains in North-Africa and Ewing (1946) did similar work on strains isolated in Algeria.

Description of Shigella:

Shigella is Gram negative, non-motile bacillus belonging to the family Enterobacteriaceae (Cowan and Steel, 1974). It produces acid from a number of carbohydrates except lactose; it is methyl-red positive and Voges-Proskauer negative. It does not grow in Koser's citrate medium, does not hydrolyse urea; deaminate phenylalanine; blackens kliglers hydrogen sulphide medium. It grows in Moller's cyanide medium; utilizes malonate; oxidizes gluconate and liquefies gelatine; however does not form lysine decarboxylase.

There are four species of dysentery bacilli. They are designated A,B,C,D (Topley and Wilson, 1983).

These are described as follows:

(1) Shigella dysenteriae (Group A): The dysentery bacteria making up this group are set apart by their inability to ferment mannitol. S.dysenteriae is immunologically heterogenous, made up of ten sharply separated serotypes arbitrarily designated by numbers. The serotypes appears to be unrelated antigentically except for unilateral cross reaction between some strains of types 2 and 6, and some strains of types 3 and 5. S. dysenteriae type 1 was the first of the dysentery bacilli to be described and it was referred to as Shiga's bacillus (Shiga, 1898). It has marked toxicity for man and experimental animals due to the formation of toxin. This has an effect upon the nervous system of experimental animals and has as an affinity for the gastro-intestinal tract (Keusch el al., 1972a). S. dysenteriae type 2 differs in that it produces *indole and ferments sorbitol and rhamnose. Other* serotypes which are culturally identical with S. dysenteriae but immunologically unrelated are known as the Sachs group of dysentery bacilli and are

designated S. dysenteriae types 3,4,5,6 to 10 (Sachs, 1943).

(ii) Shigella flexneri (Group B). This is composed of those microorganisms formerly referred to as Shigella paradysenteriae Flexner, and is now named S. flexneri serotypes 1-6 (Edward and Ewing, 1972). Members of this Group are related one to another through the possession of common group antigens but each serotype contains a type of main antigen by which it may be identified. Mannitol is usually fermented by members of subgroup B. They may be differentiated from member of subgroups A and C by means of some biochemical reactions as well as serological analysis

(iii) Shigella boydii (Group C). These are also mannitol fermenting dysentery bacilli closely resembling S. flexneri in biochemical characteristics but differ in several important aspects and are unrelated serologically. There are fifteen serotypes of Shigella boydii recognized (Edward and Ewing, 1972).

(iv) Shigella sonnei (Group D): These are late lactose fermenting dysentery bacilli. The Sonnei bacillus ferments mannitol and does not produce indole; it is serologically distinctive and homogenous.

S. sonnei can occur in two phases, namely; Phase I and II or S and R forms. The serological properties of antigens of R forms differed from those of S forms and an R or form II antiserum will not react with the S or form I (Edward and Ewing, 1972).

Shigella infections:

The Shigellae were previously thought to be highly host adapted, naturally infecting only humans and certain subprimates like chimpanzee and monkeys (Keusch, 1979). However, cases of Shigellosis have also been reported in dogs (Doems and Mortelmans, 1955); swine and calf (Ueda et al., 1963); poultry (Nyaga et al., 1985) and pigs (Ibu et al., 1987). In most instances the epidemiology of Shigellosis is traceable to a human host, whether symptomatic or asymptomatic and the route describe a circle from the anus to the mouth and back to the anus once again (Christie, 1968; Grady and Keush, 1971). In the case of S. dysenteriae 1, as few as 10 to 100 bacteria suffice to produce symptomatic disease in 10 to 40 percent of adult volunteers respectively (Levine et al., 1973). As a result of this miniscule inoculum it is rather simple

for Shigeallae to spread by contact without interposition of a vehicle such as food, water or drink to amplify the infection doses (Keusch, 1979). A thin veener of infected faeces on the fingers may be all that is required and indeed viable Shigellae can regularly be cultured from the fingers hours after experimental inoculation of bacteria on the skin (Christie, 1968). Transmission is enhanced however, when sanitation is poor or there is opportunity for the organism to reach food or water. Nonetheless, in more developed countries, epidemic shigellosis can be traced to faulty water supply or sewage (Hardy and Watt, 1948) or contamination of food as varied as milk, cheese and fruits (Christie, 1968). In those countries, Shigellosis is often an endemic contact-spread disease affecting children under the age of ten years (Keusch, 1979).

Epidemiologically, there has been a noticeable change in species prevalence over the past hundred years. The outbreaks which occured in Japan early in this century was due to S. dysenteriae 1 (Kostrewski and Stypulkowska-Misiurewicz, 1968). In the periods between WorldWars (1920-1939) this picture gradually changed and

S. sonnei emerged as the most common isolate in Europe and Japan (Reller et al., 1969). Of late S. dysenteriae has once again caused epidemic disease in many parts of the world such as Bangladesh (Rahaman et al., 1975).

Clinical manifestation of the disease:

Oral infection with Shigella generally results in the onset of fever and watery diarrhoea within twenty-four and seventy-two hours (Dupont et al., 1969). In many instances this is followed by dysentery syndrome, including abdominal cramps, tenesmus and bloody mucoid stools (Dupont et al., 1969; Mata et al., 1970). Whenever diarrhoea is detected in experimental Shigellosis; the jejunum is in a secretory state (Keusch, 1979); whenever there is dysentery, the colon is markedly inflammed and many invading bacteria are present (Takeuchi et al., 1968). Thus shigellosis appear to be a two-organ disease - a proximal bowel secretory diarrhoea and an acute bacterial colitis resulting in dysentery (Keusch, 1978).

Pathogenesis:

Enteric pathogens are known to cause disease by one or two distinct mechanisms: elaboration of enterotoxin or epithelial penetration (Sprinz, 1969).
Enterotoxin of Shigella dysenteriae was first described in 1903 and called Shiga neurotoxin (Keusch and Jacemicz, 1975). Parenteral injection of bacterial lysate or cell-free media supernatant into susceptible animals caused characteristic limb paralysis which led to the classification of Shigella toxin as neurotoxin (Olitsky and Kligler, 1920). The symptoms produced by neurotoxin were unlike the disease induced by intact bacteria in man (described above). Thus the concept that neutrotoxin participated in the pathogenesis of Shigella fell into disfavour.

It was discovered later that S. dysenteriae produced a protein toxin capable of causing intestinal fluid secretion (Keusch et al., 1972). This discovery opened the question of the role of toxins in the pathogenesis of Shigella diarrhoea. Eiklid and Olsnes (1983) compared the biological activities of the enterotoxin of S. dysenteriae with that of a well studied 20-year-old partially purified

preparation of neurotoxin from same organism. They found there were:

i) intestinal fluid accumulation

ii) limb paralysis in mice and

iii) cell death in both preparations

When the toxin was further subjected to Sephadex gel filteration, chromatogprahy, isoelectric focusing and poly acrilamide gel electrophoresis, two fractions which were cytotoxic to heLa cells were obtained. One of the fractions was also associated with enterotoxigenicity and neurotoxicity. These data suggest that Shigella enterotoxin and neurotoxin are closely related proteins and may indeed by indentical (Koo and Peterson, 1982, Eiklid and Olsnes, 1983). Other properties of Shigella toxin include its heat-liability, destruction by proteolytic enzymes; isoelectric pH 7.2 and molecular weight of 72,000 daltons (Keusch and Jacewizs, 1975; McIver et at., 1975) Two other species of Shigella (flexneri and sonnei) have been shown to produce indentical enterotoxin (Keusch and Jacewicz 1977a).

Role of Shigella toxin:

Opinions seem divided on the actual role played by Shigella toxins in the pathogenesis of diarrhoea. The toxin is lethal to mice and rabbits (Van Heyningen and Gladstone, 1953). Moreover it is cytotoxic for eukaryotic cells, such as HeLa cells (Keusch et al., 1972b) KB (human epidermoid carcinoma), normal human liver and monkey (Vicari et al., 1966). Furthermore, purified Shigella toxin inhibit protein synethesis in intact HeLa cells (Brown et al., 1980) as well as in cell free translation system (Thompson et al., 1976; Olenick and Wolfe, 1980). It has been shown that the most provocative biologic activity of the toxin is its ability to cause transudation of fluid in rabbit illeum (Keusch et al., 1972a). Since this model has proved so useful for definition of pathogenicity of diarrhoea producing strains of V. cholerae and E. coli (De and Chatterjee, 1953; Moon et al., 1970), it was logical to suggest that Shiga toxin is an enterotoxin capable of causing human diarrhoea in a manner analogous to cholera and E. coli toxins. However this view was contrary to the results of studies in animals and volunteers. Levin et al. (1973) demonstrated

that large number of non-invasive but enterotoxigenic Shigella strains were well tolerated by human models when fed orally. In contrast, the same workers showed that an invasive but non-toxigenic strain caused Shigellosis in monkeys and human volunteers. Also Flores et al. (1974) and Steinberg et al. (1975) demonstrated that Shigella enterotoxin applied in vivo and vitro into intestinal preparations did not produce any marked increase in adenylate cyclase or cyclic AMP as noted after the application of V. cholerae enterotoxin. The consensus of opinion was that the ability of Shigella organism to penerate the intestine was the primary determinant of virulence.

Bacterial invasion:

Many investigators have argued in favour of bacterial invasion of small intestinal wall as an essential process in the pathogenesis of Shigella diarrhoea (Formal et al., 1972; Levine et al., 1973). It is known that piliated strains of Shigella flexneri adhere to human intesitinal epithelial cells better than non-piliated strains (Duguid and Gillies, 1957). Also the presence of a mucosal adhesin has been implicated in the adherence of S. flexneri to guinea pig intestinal cells (Nuchamowitz and

Mirelman, 1982). In experimental Shigella and Salmonella infection, electron microscopy has shown that the brush border was destroyed and the bacteria were engulfed, by an invagination of the cell membrane of the host cells. From the vacuoles in the epithelial cells the organisms invade and destroy adjacent cells producing ulceration and local inflammation (Takeuchi et al., 1965 and Takeuchi, 1967).

Evidence reviewed by Formal and Hornick (1978) suggested that invasion, at least in Shigella, is determined by multiple chromosonal genes.

A major problem in the study of invasive organisms is the lack of a practical assay system. In the standard in vitro test (Sereny) organisms are instilled into the conjunctival sac of guinea pigs. Invasion of the corneal epithelial cells produce keratoconjunctivits which correlates with intestinal invasion (LaBrec et al., 1964). Invasion of HeLa monolayer has been used as a more practical assay, but organism adhering to the cells make interpretation of results difficult and the assay has fallen into disuse (Keusch, 1979).

Human volunteer experiments and challenge of laboratory animals are alternative techniques but of limited value in screening large number of isolates.

Pathology of Shigella infections:

Bacillary dysentery is an infection localized in the alimentary tract (Burrows et al., 1968). Histological examination of infected guinea pig illeum revealed the following changes: markedly altered villi, severe acute inflamation of the mucosa with haemorrhage, discharge of goblet cells and denudation of surface epithelium (Keusch et al., 1972a; Steinberg et al., 1972; Levine et at., 1973). Protoscopic examination of the rectal mucosa demonstrates significant colitis manifested by inflammed oedematous mucosa with patechial haemorrhages and mucus (Levine et al., 1973; Mathan et al., 1986). Rectal biospy revealed polymorphonuclear infilteration, goblet cell depletion, microulcers, haemorrhage and oedema (Levine et al., 1973; Annond et al., 1986).

Treatment therapy in Shigellosis:

In the early 1940s the sulfonamide were used for the treatment of bacillary dysentery. However, as early as the mid 1940s several countries began to note that a large proportion of isolates of Shigellae were resistant to sulfonamides (Cheever, 1952). As a result of the emergence of drug resistance the sulphonamides became ineffective in the treatment of bacillary dysentery in Japan by 1959

(Watanabe and Fukasawa, 1960; Anderson, 1965; Datta, 1965; Weissman et al., 1973). A study of almost 1,500 cases in Korea in 1953 showed that almost all strains of Shigella were resistant to sulfonamide thus indicating that the emergence of drug resistance is world wide (Garfinkel et al., 1953).

Other antibiotics including the tetracyclines and ampicillins were introduced for the treatment of bacillary dysentary (Haltalin et al., 1967). Ampicillin therapy resulted in bacteriological clearance of stool in over 90 percent of patients within 48 hours. Multiple drug resistance to ampicillin was recognised in the early 50s in Japan and in vitro transfer of R-factors from E. coli to Shigella was demonstrated (Watanabe, 1963; and Farrar and Eidson, 1971).

As result of this development, the hitherto reliable ampicillin fell out of choice for Shigellosis in many areas of the world. When isolates were increasingly resistant to ampicillin, trimethoprim-sulphamethoxazole was prescribed (Keusch, 1979). Nalidixic acid is also effective in vitro and in general in vivo but less so than the above drugs (Keusch, 1979). Again reistance to nalidixic acid has been reported (Panhotra et al., 1985).

Generally, antimicrobial therapy follows recognized guideliness for all infectious diseases: the organism should be sensitive in vitro, the infection should be responsive in vivo and the risk of side effects should be minimal compared to the expected benefits and certainly no greater risk if the disease was not treated (Keusch, 1979). An additional criterion is that the drug must be absorbable in order to reach the population of organism within the lamina propria of the gut (Haltalin et al., 1967). The guidelines ruled out the use of oral neomycin or furazolidone (non-absorbable) (Haltalin and Nelson, 1972), chloramphenicol (potentially too toxic), suphadiazine or or other sulpha drugs (resistant in vivo) (Haltalin et al., 1976).

Fluid and electrolyte:

Rahaman et al., (1975) observed that Shigella diarrhoea is not usually accompanied by massive loss of fluid leading to severe dehydration, but, however, there is significant fluid losses, requiring attention to the state of dehydration. Mild to moderate dehydration should be easily managed with oral rehydration solutions, consisting of electrolytes and an actively transported sugar such as

glucose (Keusch, 1979). The principle involved is that of co-transport of sodium equimolar with active transport of sugar (Field, 1977). Controversy still reigns regarding the specific nature and concentration of salts and sugar to be chosen. Keusch (1979) based on considerable experience in the field recommended a single ad libitum oral rehydration solution (in mmol/1: Na 81, Cl-71, HCO_3 28, Glucose 139) for treatment of all dehydrating infectious diarrhoea, regardless of stool sodium concentration, so long as physiological regulatory mechanism (thirst, renal response) remain intact, unless glucose intolerance is present. The electrolyte composition suggested for oral rehydration can be achieved in the household by mixing hald a teaspoonful of table salt, half a teaspoonful of bicarbonate of soda, a quarter of teaspoon of potassium chloride and four teaspoonful of table sugar (Glucose) in a a litre of water (WHO Diarrhoeal Disease Control (CDD) programme, 1980). For some time, the WHO Programme for the control of diarrhoeal disease (CDD) has been researching into a more stable oral rehydration salts (ORS) formula. Laboratory tests have shown that this was achievable by replacing the sodium bicabonate in the formular with trisodium citrate hydrate

(WHO Diarrhoeal Disease Control (CDD) Programme, 1984).

SALMONELLAE

The Salmonella group was originally created by medical bacterialogists to include organisms that gave rise to a certain type of illness in man and animals and were related antigentically. It is now customary to lay emphasis on biochemical activity (Topley and Wilson, 1983). Generally they are motile, produce acid and gas from glucose and mannitol, and usually from sorbitol. They rarely ferment sucrose or adonitol, and rarely form indole. Acety-methylcarbin ol is not formed, do not hydrolyse urea or deaminate phenylalanine, produce H_2S actively and grows in citrate medium and form lysine decarboxylase. The many serotypes in the group are closely related to each other by somatic and flagella antigens and most strains show diphasic variation on flagella antigens. They are pathogenic for man and many species of animals giving rise to enteritis and typhoid-like disease (Edward and Ewing, 1972).

Salmonellae are divided into four subgenera. The first of these includes the typhoid and parathyphoid bacilli and other animal types while the remaining three sub-genera comprise of organisms that are in the main parasites of cold blooded animals. (Topley and Wilson, 1983).

Sub-genera I: Most ferments dulcitol but not Lactose or salicin, KCN sensitive and do not liquefy gelatin.

Sub-genera II: Also ferment dulcitol but liquefy gelatin.

Sub-genera III:(Arizona gp): Acidify lactose but not dulcitol and liquefy gelatin.

Sub-general IV:Members acidify salicin but not lactose or dulcitol. Liquefy gelatin and resistant to KCN.

Salmonella Infections:

The epidemiology of Salmonellae other than those causing typhoid and paratyphoid fever with respect to infection in man mostly involves the animal reservoir and transmission through food in which multiplication may take place (Edel et al., 1981). Large scale or intensified farming practices confine animals or fowls in close quarters. Frequently the animals have to live in contact

with their own excreta, which also may contaminate their feed or drinking arrangement. Salmonellae, inadvertenly introduced into a herd of flock through contaminated feed, water, pasture, sub-clinically infected new stock or sometimes by rodents, wild birds or even man himself rapidly spread by cross infection (Edel et al., 1981). The excrement through effluents and through being used as fertilizer carries the contaminant to the environment and surface waters while at the abattoir their infecting Salmonella can be transfered to meat destined for human comsumption (Richardson, 1975; Turnbull, 1979).

Typhoid and paratypoid fever occur throughout the world (Bockemuehl, 1976). Endemic disease is prevalent in many countries of the Far East, Middle East, Central and South America and Africa. This is largely a reflection of the standard of water supply and sanitation. In the rest of Europe and North America the incidence of enteric fever has declined steadily in the last half century, and the disease has largely become an imported one (Mandal, 1979).

Transmission: S. typhi and S. paratyphi are strict human pathogens and the ultimate source is almost invariably man (Burrows et al., 1968). Tramsmission is through direct or

indirect contact with faeces or urine of a patient or a carrier (Bennet, Jr. and Hook, 1959; Yoshikawa et al., 1980). The principal vehicle of spread are contaminated water and food. Water has always been a dominant vehicle of typhoid spread and many large out-breaks have been water-borne;these tend to be explosive because of the simultaneous inolvement of a large number of people drinking from the same source (Mandal, 1979).

Paratyphoid outbreaks on the other hand are much less likely to be water-borne. This is because typhoid bacilli can initiate infection in small numbers (Blaser and Newman, 1982) whereas paratyphoid infection needs a higher infecting dose which is unlikely to be found in drinking water unless there is heavy polution (Mandal, 1979). Foods are common vehicles for both typhoid and paratyphoid infections. Raw fruits and vegetables are important vehicles in some tropical countries where use of human faeces for manuring vegetable crops is a common practice (Bennett, Jr. and Hook, 1959; Mandal, 1979). Unpasteurized milk and milk products such as diary cream and ice-cream have been involved in many outbreaks (Blasser and Newman, 1982). In tropical countries,

carriers among the public food handlers dealing with high-risk food and beverage such as cold dishes, fresh fruits and cold drinks play an important role in maintaining the endemicity of infection (Cvjetanovic, 1976; Onile and Odugbemi, 1987). Incidence of contamination of "fast food" has also been documented in Nigeria (Alonge and Oyekole, 1982).

The classical symptoms of Salmonella infection has been described (see Introduction). However, symptoms are also related to the severity of infection, the sites of action of Salmonellosis has been associated are malignances, liver disease, malaria, bartonellosis, Schistosomiasis and haemoglobinopathies (Bennet, Jr. and Hook, 1959). The incidence of complication of Salmonella infection include arthritis and bacteremia (Torrey et al., 1985); osteomyelitis (Wu et al, 1985); pneumonia (Canney, 1985); and endophthalamitis in infants (Appel, 1986). Other complications of enteric fever are intestinal haemorrhage and perforation (Mandal, 1979).

Enterotoxin production by Salmonella:

Despite the progress made in the study of enteric infections, the mechanism by which Salmonella causes

gastroenteritis still remains an enigma to many investigators. Koupal and Deible (1975) demonstrated the presence of enterotoxin in culture filterates of S. enteritidis. In subsequent years the enterotoxin was demonstrated in other species of Salmonella (Sandefur and Peterson, 1976; Thapliyal and Singh, 1978). Salmonella toxin has been shown to cause cell detachment and inhibited protein synthesis in intact Vero cells (Koo and Peterson, 1982). Maximum toxin was produced at pH 7.3 after 18h aerated incubation at 37 C in S. enteritidis (Sobeh and Vadehra, 1983), and evoked fluid secretion in the rabbit ileum (Giannella et al., 1973).

Pathology of Salmonella infections:

Rectal biopsies of patients with Salmonella infections showed abnormalities ranging from mucosal oedema and hyperaemia with or without petechial haemorranges to mucosal friability with slough formation and spontaneous haemorrhages (Day et al., 1978). There was dilation and congestion of capillaries in the mucosa and submucosa and focal collections of polymorphonuclear leucocytes were present in the lamina propria and in the lumen of mucosal capillaries.

In most of the biopsies, crypt abscesses and variable destruction of the crypts associated with mucus depletion were seen (Mandal, 1979).

Treatment of Salmonellosis:

For the past thirty years chloramphenicol has been the most successful drug in the treatment of enteric fever. The recommended dose in adult is 500mg every four hours till defervescence, then 500 mg six-hourly, the total lasting for two weeks (Mandal, 1979). The drug is generally given orally but in the initial stage, when the patient is frequently anorexic and perhaps having diarhoea, the drug should be given intravenously (Synder et al., 1976). However, chloramphenicol is not the ideal drug because of the risk of marrow toxicity and the high relapse rate (Kamat, 1970). Moreover, recent wide-scale emergence of S. typhi strains with R factor-mediated resistance to Chloramphenicol has brought about a new sense of urgency in the search for a suitable alternative (Mandal, 1979).

Such alternatives are:

(i) Ampicillin: During the sixties, several studies (Robertson et al., 1968; Herzog, 1976) showed the drug to be distinctly inferior to chloramphenicol.

The response was slow and the failure rate was as high as 30 percent. This drug is popular in Nigeria and has been known to induce resistance (Adetosoye and Rotilu, 1986).

(ii) Amoxycillin: This drug was introduced into Nigeria in 1973 and its use has given more promising results. When amoxycillin was used Pillay et al., (1975) found that fever in these patients lasted 6.8 days while it lasted 7.3 days in chloramphenicol group in a randomized comparative trial. Four of the chloramphenicol group relapsed but none on the amoxycillin group.

(iii) Co-trimoxazole: This drug has emerged as the most successful challenger to chloramphenicol as the drug of choice in enteric fever. After the original report by Akinkugbe et al., (1968) of more rapid defervescence with co-trimoxazole than with chloramphenicol, the drug has been used successfully in the treatment of typhoid fever in well over thousands of cases. It was later found that "toxic crisis" which may sometimes complicate chloramphenicol treatment did not occur with co-trimoxazole (Kamat, 1970).

(iv) Furazolidone: This nitrofurantoin derivative has been used mainly in the Indian subcontinent (Mandal, 1979). It has the advantage Of being cheap but appears to be somewhat less effective than chloramphenicol in man (Herzog, 1976). It has however been used to control experimental Salmonella gallinarium infection in chicken (Ojo and Adetosoye, 1977).

Isolation and characterization of Shigellae and Salmonellae

A number of suitable cultural techniques have been developed over the years for isolation of Salmonella and Shigella. For instance there is a wide acceptance of the value of elevated temperature enrichment for isolating Salmonella (Georgala and Boothroyd, 1964; Spino, 1966). Banfer (1971) found 43 C to be optimum incubation temperature and selenite F broth a suitable enrichment medium. Livingstone (1965) found that incubation of Salmonella-Shigella plates (SS agar Difco No. 0074) at 40 C allowed Salmonellas and Shigellas to form colonies but controlled the growth of unwanted organisms.

It is well known that Salmonella and Shigella are not uniformly distributed through contaminated material and invariably in enrichment cultures and that several subcultures, all made at the same time, may be necessary to demonstrate them (Cowan and Steel, 1974). Harvey and Price (1979) subcultured, after 24 hours incubation, a Selenite broth inoculated with faeces onto eight separate plates of Brillant green and MacConkey agar.

Only two out of the eight plates were found positive for Salmonella after incubation. The importance of subculture timing from fluid media has been increasingly recognized, for Grumnet (1975) found that it was no advantage to use enrichment periods longer than 24 hrs. At 24h incubation 60% of his samples were positive however, subculturing at 48h and 96h decreased the isolations rate to 23% and 11% respectively.

The problem of infection with multiple serotypes in man has been recognized (Harvey and Price, 1979). Multiple serotype infection is also of interest to Veterinary bacteriologists (Edward and Brunner, 1946). Although multiple infections are not common in Shigellosis as in Salmonella, a number of techniques have been found to be useful in dealing with this problem. These include the use of agglutinating serum. Bailey and Laidley (1955) used the method for the separation of single Salmonella serotype from a mixture by use of O and H agglutinating sera in plates of semi-solid agar. The usual method of obtaining pure cultures by dilution of a mixed culture may also be applied to material containing multiple

serotypes (Cowan and Steel, 1974). If a sample is divided into many subsamples, it is probable that the serotypes isolated from the subsamples will not be identical. Identical result can be achieved by multiple subculture from enrichment media.

The earliest reference to an enrichment method was found in the work of Vincent (1890) who used five drops of 5% (w/v) phenol in 10ml of broth. This was inoculated with 5-10 drops of sewage-polluted water and incubated at 42 C. The point was made by Thomson (1955) who recorded that ordinary nutrient broth was generally able to act as enrichment medium for the isolation of pathogen in faeces. Selenite F broth did not come into general use until Leifson (1936) developed several fuild media containing different concentration of sodium hydrogen selenite. It was found that 0.8% Sodium Selenite yielded optimum result for the isolation of Salmonella typhi. Sodium tetrathionate broth (containing a mixture of sodium thiosuplhate and iodine) is also very popular as an enrichment medium (Edward and Ewing, 1972). Knox et al., (1943) had expressed the formulae they studied

in terms of ml of 0.5 mol/1 iodine and 1 mol/1 thiosulphate to 100ml of medium. Between 1923 and 1957, there was a series of media used with a concentration range of sodium tetrathionate varying from 0.015 - 0.039 mol/1. To increase the complexity of the situation, other workers have further modified tetrathionate media by addition of novobiocin and sodium laurylsulphate (Harvey and Price, 1979).

Over the years, a series of enrichment broths have been described. This include magnesium chloride, malachite green (Rappaport et al., 1956); Strontium chloride and Strontium selenite (Chau and Huang, 1971); Bismuth sulphite (Gell et al., 1945); Gram negative (GN) broth (Hajna, 1955) e.t.c. Obviously the choice of enrichment media to be used will be determined by the specialist interest of the bacteriologist. Selenite F and GN broth were found to be essential for the diagnosis of typhoid fever and shigellosis (Harvey and Price, 1979).

For selective plating a number of selective agar are in current use. Among them are Brilliant green agar; bile salt agar, Bismuth sulphate agar, Salmonella - Shigella (SS) agar, MacConkey agar,

Hektoen enteric agar, Xylose-lysine deoxycholate agar, and Deoxycholate citrate agar. A comparative study carried out by Goyal et al., (1981) demonstrated the superiority of Xylose-lysine deoxycholate agar and MacConkey agar in the isolation of Salmonella and Shigella. Devenish et al. (1980) developed a new medium called novobiocin-brilliant green-glucose (NBG) agar for the isolation of Salmonella spp. According to them, the distinct advantage of NBG over the conventional media is the "consistent differential reaction of all Salmonella subgroups including biochemically atypical strains".

In the final analysis the choice of optimum selective agar is made on common sense grounds. If it is important to have a medium capable of growing Shigella, deoxycholate citrate (DCA), Salmonella-Shigella (S-S), Xylose-lysine deoxycholate, and Hektoen enteric agar must be used. If the bacteriologist is only interested in Salmonella typhi, Bismuth sulphite agar would be mandatory (Harvey and Price, 1979). Also addition of 1 ug/ml potassium tellurite to MacConkey agar is known to serve as secondary enrichment medium and facilitate the isolation of Shigellae.

Differentiation and Characterization of Salmonellae and Shigellae

Generally, the differentiation and characterization of enteric bacilli is based upon a variety of biochemical and cultural reactions and upon antigenic structures (Cowan and Steel, 1974). A useful primary differentiation is made on the basis of lactose fermentation which is roughly correlated with pathogenicity. The coliform bacteria ferment this sugar rapidly with the formation of acid and gas in 24h, while Shigellae and Salmonellae (essential pathogens) do not ferment it. Similarly, the dysentery bacilli or Shigellae divide into two groups on the basis of fermentation of mannitol and are anaerogenic (Edwards and Ewing, 1972). While the Salmonella group in general produce gaseous fermentation with the exception of the typhoid bacilli.

A great variety of cultural reactions, not only the conventional sugar fermentations, formation of indole from tryptophane etc but various kinds of specialized tests such as utilization of tartaric acids and malonate and amino acid decarboxylase activity have been useful in the physiological characterization of the Salmonella and Shigella group (Edwards and Ewing, 1972).

Serology:

The technique of antigenic analysis have been developed in the study of *Shigella* and *Salmonella* groups and the antigenic composition of these groups is perhaps better known than any other bacteria (Cowan and Steel 1974). Three types of antigens are present in the Salmonella group, two are associated with the cell substance (somatic or O antigen and V or virulence antigen) while the third is the flagella or H antigens. Partial or complete identification of serotype is carried out by the use of antisera containing appropriate antibodies. The serological differentiation of *Salmonella* strains is called Salmonella typing and requires a lot of skill and specialty.

In case of *Shigella* there are no flagella antigens and the serotyping is relatively easy. However, in the past the nomenclature of the dysentery bacilli has been somewhat casual, and informal names such as Shiga bacillus, Flexners bacillus, Strong bacillus and the Hiss-Russel Y bacillus had wide currency (Edwards and Ewing, 1972). The name Shigella for the genus has

gained reasonably wide acceptance. International agreement has also been reached based on species name and serotypes as suggested by Ewing (1958) to introduce a well-come order and stability.

Antibiotics

Antibiotics are chemical substances obtained from bacteria or fungi which in dilute concentrations are capable of inhibiting the growth of microorganisms or capable of destroying them (Pelczar et al.,1977). The word antibiotic has come to refer to a metabolic product of one organism that is detrimental or inhibitory to other microorganisms in very small amounts (Garrod et al.,1981). Antibiotics were known by their activities long before they were given the name by which they are now called. Many years ago, the Chinese use moldy soybean curd for the treatment of boils and controlled foot infections by wearing sandals furry with mold (Pelczar et al., 1977).

The first systematic search for, and study of antibiotic (reported by Brock, 1974) resulted in the discovery of actinomyceton in strains of actinomycetes. In 1929 Alexander Fleming discovered the famous antibiotic named penicillin. Several thousand antibiotic substances have been isolated and identified since 1940 (Garrod et al., 1981). Many of them are of no practical importance yet, but a few have changed the entire concept of chemotherapy. These is no doubt that many more and possibly better antibiotics will be found (O'Brien et al., 1987).

Mode of action of antibiotics:

Antibiotics can inhibit or kill microorganisms in one of several ways (Pelczar et al., 1977).

(a) Inhibit cell-wall formation.

(b) Damage of the cell membrane (cytoplasmic membrane).

(c) Interfere with protein synthesis

(d) Inhibit nucleic acid metabolism.

A big setback to the use of antibiotics was the discovery of antibiotics-resistance phenomenon.

Antimicrobial resistance in Man:

The use of antibiotics in human and Veterinary medicine for the treatment or prevention of infections has favoured the selection of antibiotic resistant organisms and their wide dissemination in human population (Ogunnariwo, 1987). The first clinically serious consequence of antibiotic resistance to attract considerable attention was the widespread dissemination in hospital of strains of Staphylococcus aureus that were resistant to penicillin by virtue of their ability to form an antibiotic-destroying enzymes (B-lactamase), and subsequently acquired resistance to several other chemically related antibiotics (Anderson, 1968).

By the early 1970s the gram negative bacilli have replace S. aureus as the most important cause of hospital acquired septic infections, and at least in a number of developed countries, widespread endemic prevalence of multiple-antibiotics resistant strains of S.aures became uncommon (Parker et al., 1974; Adegoke, 1983). A more serious situation developed when antibiotics resistance became a common cause of treatment failure in diarrhoeal enteric disease (O'Brien et al., 1987).

Plasmid - determined transferable resistance was detected with increasing frequency among E. coli, Shigellae and Salmonellae during the 1960s, and a few years later there were widespread epidemics of severe bacillary dysentery and typoid fever caused by strains that were resistant to several antibiotics, including the agent of choice for the treatment of these infections (Barrada and Guerrant, 1980).

In recent years, pencillinase-forming gonococci have shown considerable spread world-wide (Jaffe et al., 1981; Johnson et al., 1981) and several B-lactamase plasmids have been identified among them (Ansink-Schipper et al., 1982). Pencillin-resistant

strains of pneumococci became locally prevalent in South Africa (Jacobs et al., 1978) where they caused treatment failures and death of infants. The resistance was non-enzymatic and chromosomally determined, and was often accompanied by resistance to other antibiotics including tetracycline and chloramphenicol.

Until quite recently Vibrios with R factors coding for multiple antibiotic resistance were seen infrequently. Extensive epidemics of cholera have now been reported from the United Republic of Tanzania (Mhalu et al., 1979), in which an initially sensitive strain became predominantly resistant within a few months, the R factors responsible for this indicated resistance to several antibiotics, including tetracycline the drug of choice for treatment.

Evidences have indicated that serious consequences of antibiotics resistance were no longer confined to urban hospitals but were being encountered increasingly in the general population (WHO Scientific Working Group, 1983). There is also a greater prevalence in developing countries of resistance to easily available antibiotics such as ampicillin, tetracycline, chloramphenicol and sulfonamides, than is known to

occur in developed countries (Osoba, 1979). Survey of B-lactamases in Enterobacteria from developing countries revelead a corresponding greater variety of types, and of instances of multiple B-lactamases in the same isolates (Ogunnariwo, 1987).

Antimicrobial resistance in Animals:

It is well recognized that the administration of antibiotics to animals for any purpose (growth promotions, prophylaxis or subtherapy) lead to the accumulation of resistant bacteria flora (WHO Scientific Working Group, 1983). Antibiotics have been used for all these purposes for many years and it is difficult to separate the contribution made by each of the above (three purposes) to the pool of resistant organisms in animals.

According to Edel et al. (1981) the danger of this pool is due to the fact that:

(i) Antibiotic-resistant pathogens common to animals and man may reach man by cross-infection; and

(ii) Antibiotic-resistant non-pathogenic organism in an animal may be passed to and colonize man, thereby carrying R plasmids which are subsequently transfered to human pathogens or to indigenous flora in the human body.

Many of the enteric gram negative organisms (e.g Salmonella species and some strains of Campylobacter) infect man as well as animal (Topley and Wilson, 1983). The larger proportion of Salmonella infections in man are derived from eating contaminated meat, infected egg or milk and therefore, indirectly, from animal course. Whenever strains of antibiotic-resistant Salmonellae arise in animals they eventually reach man (Rowe et al., 1979; Lyons et al., 1980).

The frequency of antibiotic resistance in various Salmonella species varies from one country to another. Linton (1981) revealed that while resistance to Sulfonamides and Streptomycin occured in up to 50% of isolates in the United Kingdom from 1958-1959, multiple resistance was rarely experienced in most species.

Where this did arise, it occured almost exclusively in S. typhimurium. A survey of animal Salmonellae in North-East USA by New et al.,(1957) revealed that the majority of S. typhimurium, S. saint-paul and S. heidelberg were resistant to three or more antibiotics including ampicillin, kanamycin and tetracycline in addition to sulfonamides and streptomycin. Several

authors (Lyons et al., 1980; Edel et al. 1981) attributed this resistance at least in part, to the continuing widespread use of antibiotics as feed additives and this may be a significant cause since antibiotics are often used at levels considerably higher than those recommended.

The antibiotics resistance status of the normal gut flora is somewhat different. The oral administration of antibiotics which is the common route of therapy, prophylaxis and growth promotion, invariably favours the selection of resistant strains particularly from among the Enterobacteriaceae and other gram negative bacilli which then become prevalent (WHO Scientific Working group, 1983). Evidences now abound confirming that these resistant strains reach man via food chain. The most definite work has been done with E. coli. The highest incidences of antibiotic resistant E. coli. were found in calves (Howe and Linton, 1976; Adetosoye, 1980a) pigs and poultry (Linton et al., 1977) in situations where antibiotics have been widely used as feed additive.

Antibiotics are now not much used in preventing food spoilage because of high frequency of antibiotics resistance developed by contaminating organisms (WHO Scientific Working Group, 1983).

Properties of infectious drug resistance:

The original discoveries in the field of transferable drug resistance were made in Japan (Anderson, 1968) and followed the detection in that country from 1957 onward of a rise of drug resistance in Shigellae. Most of the early work was done by the Japanese, one of the most active being Watanabe (1963) whose review was the first comprehensive introduction of the subject. Since then however, quite a large number of workers all over the world have contributed to the knowledge in this field.

The properties of transferable drug resistance may be summarised as follows (Anderson, 1968): The resistance may be to a single drug but is usually multiple. It is more commonly found among Enterobacteriaceae that is, Shigella, Salmonella Escherichia coli, Klebsiella, Proteus and related organisms inhabiting the animal or human intestines.

The genetic elements controlling transferable resistance are designated "R (resistant) factors" and are borne on plasmids (Watanabe, 1963).

The nature of Plasmids:

Plasmids are extrachromosomal genetic elements, they are composed of DNA and are inherited yet dispensable and have the ability to replicate independently of the chromosome (Farar, 1979). Plasmids are the physical entities responsible for transmissible drug resistance (Falkow, 1975; Mitsuhashi, 1977; Jacoby and Swartz, 1980). Structurally, they are covalently closed circular molecule of double stranded DNA. Most plasmids range in size from a few million to approximately 100 million daltons in molecular weight although a few are larger or smaller (Jacoby and Swartz, 1980). They are thus about one percent as large as the bacterial chromosome which they resemble in general organization (Farrar, 1979; Jacoby and Swartz, 1980). Plasmids harbour genes which can be divided into four categories (Jacoby and Swartz, 1980): essential genes, genes involved in plasmid transfer (tra-genes), genes

concerned with interactions with other self replicating genetic units or replicons, and genes that affect the host's cell interaction with the environment. The latter genes include those coding for enterotoxin production, haemolysins, surface adherence antigens, resistance to heavy metals, ability to ferment certain carbohydrates and various other functions.

For some plasmid systems, the resistance and transfer genes occur on separete molecules, each capable of independent replication. The transfer of such an aggregate multimolecular plasmid is achieved by mobilization of the Tra-R plasmids by its Tra partner (Anderson, 1968). In other plasmids, the resistance genes and transfer functions are combined in a single molecule which may or may not be able to dissociate into separate replicons.

Plasmid detection:

Plasmids can be detected by physical or genetic techniques. A general method is centrifugation to equilibrium in Ceasium chloride gradients containing the dye ethidium bromide which binds differentially to plasmids and chromosomal DNA to allow their separation (Helinski and Clewell, 1971). Recently, much simple

and more rapid techniques for plasmid visualization have been developed using agarose gel electrophoresis (Meyers et al., 1976).

Chromosomal or plasmid mediated resistance:

Davies and Rownd (1972) suggested that micro-organisms acquired resistance to only one drug at a time and that organisms resistant to several drugs had arisen by accumulation of successive mutations. However, mutation and selection could not explain the rapid increase in this incidence of drug resistance especially in Shigellae which show resistance to as many as four to six drugs. Transfer of multiple drug resistance in vitro by mixed cultivation of naturally occuring multipe drug resistant strain with drug sensitive ones have proved conclusively that multiple drug resistance was due to the presence of extrachromosomal resistant determinants or R factors (Watanabe and Fukasawa, 1961a).

Other plasmids act as fertility factors to promote exchange of chromosomal genes or production of Bacteriocins, metabolic enzymes, enterotoxins, virulence factors, resistance to heavy metals, to ultraviolet radiations and production of haemolysins (Jacoby and Swartz,1980).

Methods of plasmid transfer:

Generally, there are three methods of genetic transfer of information in microorganisms, cell to cell contact or conjugation, transformation (naked DNA mediated) and transduction (bacteriophagemediated) Watanabe, 1966; Lacey, 1971). Conjugative plasmids can be detected genetically by testing for transfer of the property they determine to a suitable recipent by mating (Falkow, 1975). For example, to detect R-plasmid transfer to E. coli, an antibiotic-sensitive recipient made chromosonally resistant by mutation to nalidixic acid is mixed in liquid culture with the presumptive donor organism and then plated on media containing nalidixidic acid (which will prevent growth of donor) together with an antibiotic that prevents growth of recipient except resistance transfer occurs. In this process pair formation takes place between donor and recipient cells by means of a specific structure synthesized by the donor, the sex pilli. This attaches to the surface of the recipient cell and may serve as the conduit through which plasmid DNA passes from donor to recipient

(Anderson, 1968). There are also "aggregates" consisting of a number of bacteria joined together by sex pilli. One strand of plasmid DNA passes into the recipient cells and complimentary strands are synthesized in both donor and recipient cells (Dunny et al., 1978).

In transformation a drop of a solution of transforming DNA from donor strain is mixed with the recipient cells. The treated cells are then spread on antibiotics-containing agar plates for selection (Ogunnariwo, 1987).

Transduction procedures employ bacteriophages e.g. phage epsilon in Salmonella in carrying out genetic transfer from donor to recipient (Watanabe and Lyang, 1962) and phage P1 in E. coli (Harada el at., 1963).

Conditions governing the frequency of Transfer in conjugative plasmids:

The frequency of transfer of drug resistance is the resultant of the interaction of a number of influences (Anderson, 1968; Davies and Smith, 1978):

(a) The Recipient Strain: Different members of the Enterobacteriaceae vary in their susceptibility

to infection with a given R factor. In the case of R factors in which the determinants are closely linked with the transfer factor, this is probably largely the result of the varying capacity of different strains for accepting the transfer factor encoded (Watanabe, 1963). E. coli K 12 is known to be an excellent recipient for R-factors of diverse origin. On the other hand S. typhimurium type 36 is a relatively poor recipient for some R-factors originally found on E. coli and Shigellae but good recipient for R-factors originally defined in S. typhimurium.

(b) The donor strain: Some strains seem to be more efficient donors that others. This character is presumably dependent at least, in part on the state of the repression of the transfer factor in the strain concerned.

(c) The Physiological state of the donor and recipient cells: Transfer of R-factors to recipients cultures is usually low in frequency and may be less than 10^{-5} per donor cell after mating for one hour. If the host cells are in suitable physiological state, e.g. log phase, adequate nutrient supply, etc

spread of resistance factor is enhanced in recipient culture.

This is caused by derepresion of the transfer factor concerned. The initial derepression depends on the physiological state of the donor-recipient system. Epidemic spread of the transfer factor does not start until more than twelve hours have elapsed after mixing donor and recipient trains (Anderson, 1968). It then results in rapid spread between recipient cells, so that fifty percent or more are infected within a few hours.

(d) <u>The nature of the linkage between the determinants and the transfer factor:</u>

A reversible association of Ampicillin resistance gene and the transfer factor result in a lower rate of transfer of the complete R factor than close linkage of the tetracycline resistance genes and transfer factor type, in which the rate of transfer of the determinant is that of the transfer factor itself.

(e) Non-specific factors: For example, the density of cultures determines the frequency of contact and therefore the possibility of conjugation between donor and recipient cells and recipient cells that have already acquired R-factors. Some plasmids transfer much more efficiently on solid than in liquid medium so the mating mixture can be impringed on a filter for more efficient conjugation (Jacoby and Swartz, 1980). For a few plasmids, transfer is temperature sensitive and occurs more readily at 22 C than 37 C (Tarawaki et al., 1967). Thus both low and high incubation temperatures should be employed when attempting to detect plasmid transfer. However, at about 18 C transfer does not occur (Falkow, 1975).

Incompatibility behaviour:

Incompatibility behaviour is often used to classify plasmids genetically. Compatible plasmids can coexist in the same host, while incompatible plasmids cannot and so tend to displace each other (Chun et al., 1984).

Plasmids carrying resistance factors (R-factors) found in Salmonellae and Shigellae have been grouped as follows (Jacoby and Swartz, 1980)

Plasmid	Original Host	Characters
R_1	Salmonella paratyphi	Dissociate into conjugative resistance transfer factor RTF, r determinant, Am, Cm, Km, Sm, Watanabe and Fukasawa (1961a).
R_{100} (also called NRI and R222)	Shigella flexneri	TC or RTF; Cm, Hg, Sm, Su on r-determinant. Watanabe and Fukasawa (1961a); Davies and Rownd (1972).
R_{10}, R_{12}, R_{13} R_{14}, R_{16}, R_{26}	Shigellae	r determinants carry different marker: Am, Cm, Sm, Su Horada et al., 1983).
L-T-2	Salmonella typhimurium	r determinant: Sm, Tc, Cm, Su (Watanabe and Fukasawa, 1961).

Am = Ampicillin; Hg = Mercury ions; Sm = Streptomcyin; Tc = Tetracycline; Cm = Chloramphenicol; Km = Kanamycin; Su = Sulphonamide.

Mechanism of Plasmid-determined Resistance:

Plasmid determined resistance to antibiotics by four biochemical mechanisms (Benveniste and Davies, 1973; Davies and Smith, 1978; Jacoby and Swartz, 1980). These are:

(i) The drug is detoxified or inactivated;

(ii) Transport of the antibiotic into the cell is blocked;

(iii) The target site is altered so that binding of the drug is reduced or eliminated; or

(iv) The antibiotic inhibited step is by-passed.

The following are known mechanisms which are involved in the resistance against some of the popular drugs:

(a) Streptomycin: Streptomycin is attacked by two different enzymes viz: 3"-O-Phosphotraseferase adenyltransferase (Jacoby and Swartz, 1980). These enzymes when purified have been shown to be fairly small proteins, with submit Molecular weight of 17 to 29 x 10D (Davies and Smith, 1978). They are found in the periplasmic scape between the inner and outer cell membranes.

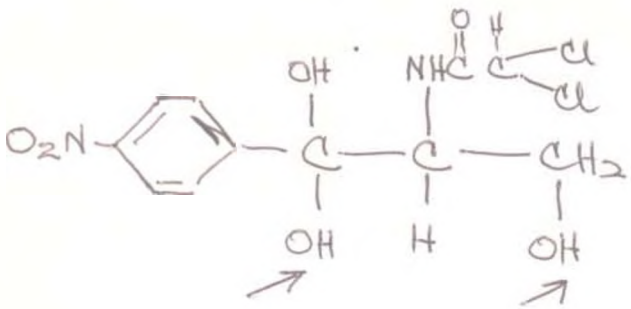
Since these enzymes permit growth in the presence of unmodified drug they are thought to provide resistance by modifying aminoglycoside at a faster rate than it can be transported into the cell (Dickie et al., 1978). Once modified the aminoglycoside is no longer able to inhibit protein synthesis.

(b) Ampicillin: A major proportion of the resistance to B-lactam antibiotic such as ampicillin, carbenicillin and cephalosporins among strains of gram negative bacilli is produced by a variety of extrachromosomal (plasmid or transposom mediated) B-lactamases, the effect of which is augmented in some strains by impaired diffusion of the antibiotic into the bacterial cell (O'Brien, 1987; Ogunnariwo, 1987). The enzymes are produced inducibly or constitutively and are cell-bound rather than excreted into the surrounding medium (Benveniste and Davies, 1973). More than twenty of these B-lactamases have been identified, but a few predominates, and one of these, the TEM-1-B-lactamase, is encountered more often than are all of others combined (Medeiros, 1984).

The B-lactamases are known to hyrolyse the B-lactam ring present in amplicillin and other related antibiotics (Ogunnariwo, 1987). The enzymes vary in molecular weight from 12 to 44.6 x 10 and, so far as they have been examined, are immunologically distinct, except for TEM-1 and TEM-2 which cross react and appear to differ by very few, perhaps, one amino acid (Ambler and Scott, 1978). The level of resistance encountered is complicated by such factors as the substrate affinity of the B-lactamase being produced, the extent of induction (if the enzyme is inducible), the location of the enzyme in the cell and the ease of entry of the antibiotic into the organism (Benvesnite and Davies, 1973).

(c) Chloramphenicol: Since the introduction of chloramphenicol, the emergence of bacteria resistance to this antibiotic has been well documented. Okamoto and Suzuki (1965) were the first to show the inactivation chloramphenicol in the presence of acetyl coenzyme A. The inactivation of chloramphenicol was due to acetylation of the

antibiotic to yield 3-acetyl and 1, 3-diacetyl esters (Benveniste and Davies, 1973).



The structure of chloramphenicol. The arrows point to the hydroxyl groups that are acetylated (Adapted from Benveniste and Davies, 1973).

The enzyme is synthesized constitutively and is located intracellularly. It has a molecular weight of 80,000 and consists of four identical catalytically inactive subunits each having a molecular weight of 20,000 (Benveniste and Davies, 1973; Jacoby and Swartz, 1980).

Only sporadic isolates of Salmonella typhi were resistant to chloramphenicol, which was agent of choice for treatment of typhoid since 1972, when there was a major outbreak of chloramphenicol resistant Salmonella typhi in Mexico (Anderson and Smith, 1972). Subsequently, strains of S. typhi similarly carrying a

chloramphenicol resistance gene on a Inc HI plasmid and similarly resistant to streptomycin, sulfonamide and tetracycline have caused an outbreak in India and have become endemic in Vietnam and Thailand (O'Brien et al., 1987). The chloramphenicol-resistance gene was also found on the InC F 1me plasmids carried by the widely epidemic non-typhoid serotypes of Salmonella (Jacoby and Swartz, 1980).

(d) Tetracyclines: Resistance to tetracycline is common in R+ enteric strains (Chopra and Howe, 1978). The fact that the gene mediating tetracycline resistance seems closely linked to that portion of the R factor which determines transmissibility may be the reason for its prevalence. Tetracycline is the only antiobitics for which the major mechanism of plasmid determined resistance is a block in drug uptake (Chopra and Ball, 1982). Sensitive bacteria accumulate tetracycline by two independent transport systems one active and one passive (McMurry and Levy. 1978). Both systems are partially inhibited in resistant cells. Unlike

most plasmid determined resistance mechanism in gram negative bacteria, tetracycline resistance is inducible (Chopra and Howe, 1978). With induction, several new proteins are made and associate with the cell memberane, where they presumably interfere with antibiotics uptake. Infact, resistant cells possess an uptake, and perhaps and efflux system for tetracycline that is lacking in sensitive cells (McMurry and Levy, 1978).

Tetracycline resistance genes are widely distributed among isolates of Enterobacteriaceae in all parts of the world (Chopra and Howe, 1978).

The levels of drug resistance confered by R-factors:

The level of resistance attained by a strain carrying R factor depends on two constituents (Anderson, 1968); the host cell and the resistance determinant. Watanabe and Fukwassa (1960) pointed out that the same R factor may promote different levels of resistance in different hosts. A streotomycin R factor conferred resistance to more than 100 ug/ml of the drug on a Shigella strain, and to only 10 ug/ml on a strain of E.coli.

Watson (1967) transfered R factors for multiple resistance, including that to streptomycin, from Shigellae to E. coli K-12. The recipient cultures showed lower streptomycin resistance than the donors. The differentin streptomcyin resistance levels were however, of a smaller order than those demostrated by Watanabe and Fukasawa (1961). In contrast, Lewis (1967) demonstrated that multi-resistance R factors endowed the E. coli recipient with higher chloramphenicol resistant than that of shigellae donors.

The minial inhibitory inhibitory concetration of ampicillin and pencillin for S. typhimurium of page type 1a resistance to A, S, Su and T described by Anderson and Datta (1957) varied between 64 and 128 ug/ml but was the same for both drugs in each wild strains K-12 infected with R factor from one of the strains of which the MIC was 128 ug/ml, developed an ampicillin MIC of 64 ug/ml, and a pencillin MIC of 91 ug/ml, the difference being reproduceable.

These observations confirm that the level of resistance confered by an R factor depends on both the R factor and the host cell. While extreme variations

from one hots to another exists, it is also common to find, in effect, the same level of resistance in the original host and in a new host.

Non-plasmid-mediated resistance in enteric bacteria

Although most multiple-drug resistance in enteric bacteria is plasmid linked, important types of resistance can be determined by genes located on the bacterial chromosome (Farrar, 1979). Non-transferable, apparently chromosome mediated cephalosporing beta-lactamases are found in many strains of Escherichia coli, indole-positive Proteus species; Enterobacter, Citrobacter, Serratia marcescens and Pseudomonas aeruginosa (Richmond and Sykes, 1973; Medeiros et al., 1984 Farrar and Newsome, 1976). Virtually all strains of Serratia marcescens possess the chromosomal cephalosporinase, and many also elaborate the plasmid-mediated TEM beta-lactamase (Farrar and O'Dell, 1976).

In P. aeruginosa, while much resistance is due to plasmid-controlled aminoglycoside - modifying enzymes, many gentamicin-resistance strains have been isolated from clinical infections which exhibit diminished accumulation of gentamycin but no detectable enzymatic modification of the antibiotic and no plasmids (Bryan et al., 1974; Bryan et al., 1976). Strains resistant by the enzymatic mechanism are usually

highly resitant(MIC> 100 ug/ml) whereas those with diminished pearmeability are less resistant to gentamcyin (MIC 6-12 ug/ml) (Farrar, 1979).

Plasmid-mediated resistance and that due to chromosomal genes may not be entirely unrelated (Farrar, 1979). Transposable elements from plasmids can insert into the bacterial chromosome and can decrease or increase the level of functional activity of genes at or near the site of insertion (Saedler et al., 1974).

For example, insertion of a transposon into the chromosome might de-repress the synthesis of a drug-inactivating enzyme controlled by chromosomal genes, leading to an increase in resistance (Farrar, 1979). The prevalence of plasmids in medically important bacteria has undoubtedly increased since the beginning of the antibiotic era, and it appears that many of these possess transposable DNA sequences.

Lost of R Factor: The foregoing observations suggest four methods whereby an R factor may loose it transferability (Anderson, 1968):

(a) Segregation of the R factor during transfer so that only resistance determinants enter the recipient.

(b) Segregation during division of the host strain so that some of the progeny receive determinants only.

(c) Transduction to a fresh host, in which the determinant only is carried over by the transducing phage.

(d) Destruction of the transfer factor by mutagens, without damage to the determinants also known as curing.

Among the latter are many compounds that appear to function through their interaction with DNA and others that have different sites of action (Jacoby and Swartz, 1980). Known curing agents include ethidium bromide, acriflavine, acridine orange, chloroquine, guinone, daunomycin and methylene blue (Hahn and Clak, 1976).

Transposons: It is known that many drug resistance genes resides upon DNA sequence which can be translocated from one plasmid to another (Cohen, 1976; and Kopecho, 1967). Such transposons have been described which specify resistance to ampicillin (Hedges and Jacoby, 1974), tetracycline (Klechnar et al., 1975) and chloramphenicol (Gottesman and

Rosner, 1975). Six of the well-established plasmid-determined beta-lactamases in gram negative bacteria and four of the novel beta-lactamases are determined by transposons (Jacoby and Swartz, 1980). These include the transposons coding for three TEM-like beta-lactamases in Pseudomonas spp. TEM-1 is determined by Tn 3 and TEM-2 by Tn 1 which shares considerable homology with Tn 3 (Ogunnariwo, 1987).

Plasmids and Virulence in Salmonella and Shigella:

It has become increasinly evident that plasmids often play an important role in the pathogenic potential of variety of microorganisms. The pathogenic potential of Shigella flexneri is directly correlated with the ability to invade and multiply within the colonic mucosa (LaBrec et al., 1964). Genetic analysis has established that virulence in S. flexneri is associated with several chromosomal loci (Formal et al 1971; Gemski et al., 1972). Sansonetti (1982) established that all invasive S. flexneri strains irrespective of serotype, harbour a large plasmid of >140 megadaltons in size.

Also spontaneous variants that have lost this 140 megadalton plasmid concomitantly became avirulent i.e. could neither invade HeLa cells or produce

kerato-conjunctivitis in guinea pigs. Similar genes are also known to be haboured by 120 megadalton plasmids (Maurelli et al., 1985). At least seven polypeptides (designated a through g) have been identified as unique products of the virulence plasmids of Shigella spp and entero-invasive E. coli (Oaks et al., 1986). Four of these polypeptides (a, b, c and d) are synethesized from a 37-kilobase fragment of a cloned plasmid DNA that has the capacity to restore HeLa cell invasiveness in a Shigella recipient which has lost the 140 MDA plasmid (Maurelli et al., 1988).

In enterotoxigenic E. coli the following plasmids have been isolated and analysed; Enterotoxin plasmids (Both St and Lt); colonization antigen plasmids; Vir plasmids (which specified a surface antigen as well as a heat labile, acid-sensitive, nondialyzable toxin lethal for rabbit, mice and chickens; and Col V plasmid (Elwell and Shipley, 1980). Literature concerning the virulence plasmids of Shigella are few. Apart from those concerned with its invasive action (mentioned above) no plasmid has been implicated in the enterotoxin production of Shigella spp (Ellwell and Shipley, 1980).

In the case of Salmonella a similar paucity of information is encountered. Possession of plasmid has not been established with virulence. Butler et al. (1979) transfered S. typhi plasmid to S. typhimurium and the virulence of the R strain for mice was assessed by intraperitoneal 50% lethal dose and by the number of organisms found in the spleen of infected animals. The plasmid containing S. typhimurim strain did not prove to be move virulent for mice. Elwell and Shipley (1980) inferred that the supposition that the epidemic R plasmids per se increased the virulence of their host bacteria lacks convincing supportive evidence. Even in a situation where the presence of DNA regions specifying virulence enhancing properties has been excluded, it is clear that in certain circumstance, R (resistance) plasmid can be considered to be virulence plasmids. The concepts of virulence, that is, the extent or degree of pathogenicity, in terms of human disease is extremely difficult to quantify due to the many contributing and interacting factors. There is no question, however that the sudden and unexpected of multiple drug resistance in enteric pathogens seriously compromised appropriate

dical treatment with the resultant high morbidity (Anderson and Smith, 1972).

Immunology of Salmonella and Salmonella vaccine:

Animal experiments have shown that it is possible to obtain a high degree of immunity in rabbits and guinea pigs against intra-peritoneal inoculation (Burrows et al., 1968). Recovery from experimental enteric infection in the Chimpanzee is also associated with an immunity to subsequent challenge by the oral route, eighteen months after the primary infection (Gaines et al., 1960). The immunity is associated with the development of humoral antibodies such as agglutinins, precipitins and the like. Lysin is also produced, and, like the Vibrio cholerae, the typhoid bacilli undergoes visible dissolution and disintegration in the peritoneal cavity of the immune animal (Burrows et al., 1968)

The Widal agglutination test has been an invaluable tool in serum diagnosis of typhoid fever (Levine et al., 1978).

The test is a macroscopic one and is carried out with both H and O antigens. The interpretation of a single such test must take into consideration

ancilliary data such as a previous immunization or attack of typhoid fever and the prevalence of endemic typhoid in the general population (Burrows et al. 1968). It is therefore difficult to set arbitrary limits; in most instances an O titre of 1:100 and an H titre of 1:200 may be regarded as significant.

Typhoid vaccine has been used for many years to produce active prophylactic immunity (Topley and Wilson, 1983). The vaccine consists of a saline suspension of typhoid bacilli, usually 1000 million per milliltre, which are killed by heat, phenol, formaldehye etc. (Burrows et al., 1968). Triple vaccine consisting of para A and B bacilli in addition to typhoid bacilli (TAB) to the same total concentration but in a ration of 2:1:1 has been commonly used (Topley and Wilson, 1983).

Questions of the assay of the immunogenic potency of typhoid vaccines, and their antigenic composition are raised perenially. There are discrepances between the results of field trials, studies of the experiemental diseases, and and immunogenic potency tests, and final answers are as yet possible. Typhoid fever remains a serious public health problem in many regions of

the world, and the typhoid vaccines which are available are not wholly satisfactory.

Germanier and Furer (1975) isolated a galactose epimerase (galE) mutant of Salmonella typhi (Ty 21a) and the result obtained with animal model indicated that this strain has the potential for use as live vaccine. Wahdan et al. (1982) carried out a field trial to check the safety and stability of the mutant strain and tested the protection against typhoid fever afforded by three oral doses. Earlier studies by Collins and Carter (1972) have shown that live attenuated vaccine when administered orally, provided mice with better protection against subsequent challenge than to inactivated vaccines.

The effectiveness of the Ty 21a vaccine was assessed by analysis of the incidence of typhoid fever in the subjects. The result of the follow-up (1 year after) indicated that in the dosage schedule tested, the Ty 21a mutant strain found previously to be stable and safe, was protective against typhoid fever for at least one year, without any side effect like fever, malaise, headache and localized reactions at the site

of inoculation. These side effects have served to put the earlier parenteral vaccines out of reckoning (Burrows et al., 1968).

The current commercially available vaccine formulation incorporates 10^9 Ty 21a strain in gelatine capsule and is given with 0.8g of $NaHCO_3$ to neutralize gastric acidity (Scientific activities WHO-OMS, 1983).

Immunity to Shigella Infection and Shigella vaccine:

The development of an immunity effective to some degree is indicated by the relative resistance of the resident population of an endemic area of the acute disease which affects recent arrival, e.g. "acclimatization diarrhoea" (Burrows et al., 1968; Steffen et al., 1988). This phenomenon is well known to residents of temperate climates visiting tropical and subtropical areas.

Antibodies, agglutinins, are found in response to infection with dysentery bacilli usually appearing after the sixth day (Topley and Wilson, 1983). The diagnostic significance agglutinins is somewhat uncertain largely because "normal" agglutinins are

common. Normal serum commonly agglutinates Shigella dysenteriae 1 in 1:20 dilution, but a titre of 1:40 or higher is suggestive of infection (Burrows et al., 1968). The antibodies are apparently unrelated to effective immunity.

Unlike infections with typhoid and related bacilli, bacillary dysentery remains a localized infection, and the bacilli rarely penetrates beyond the regional lymphatics at the most (Topley and Wilson, 1983). Attempts to immunize humans or other primates with killed vaccines or even virulent organisms, administered by the parental route have been met with very little success. Several kinds of living vaccines administered by the oral route have been used. One is made from bacilli which is streptomycin dependent and therefore unable to multiply in the absence of the antibiotic (Formal et al., 1965). Such vaccines of S. flexneri have been field-tested in Yugoslavia and have been found to give significant, but type-specific , protection against the naturally occuring infection (Mel et al., 1965).

Polyvalent vaccines of hybrid strains of S.flexneri 1b, 2a, and 3 and S. sonnei 1 given in two doses by oral route have been shown to produce a highly effective immunity against challenge with the virulent strains. Formal et al. (1981) transfered plasmid reponsible for form 1 antigen synthesis of S. sonnei conjugatively to established gal E S. typhi strain. Serological studies revelead that the derivative strain produced the form 1 antigen in addition to the normal S. typhi somatic antigen. Testing in mice demonstrated that the derivative form 1 gal S. typhi strain is protective against both S.sonnei and S. typhi challenges. It was therefore suggested that gal E S. typhi 21a oral vaccine strain, may also serve as a useful carrier for other antigenic determinants to protect against Shigella infections.

CHAPTER THREE

ISOLATION AND CHARACTERIZATION OF SHIGELLAE AND SALMONELLAE INVOLVED IN DIARRHOEA

INTRODUCTION

Salmonella and Shigella isolation and characterization is a dynamic study and progress in this area continues to be made. Both organisms are found in the intestinal tract of man and other warm-blooded animals as commensals of limited pathogenic potentials associated with diarrhoeal disease (Topley and Wilson, 1983).

Over the years a number of procedures have been developed to aid quick isolation and identification from faeces. The importance of pre-enrichment of suspected materials as a first step in the isolation of these organisms have always been emphasized. A number of enrichment broths have also been described (Harvey and Price, 1979). For Salmonella enrichment the following have been used: Magnesium chloride, malachite green, strontium chloride and strontium selenite, Gram negative (GN) broth, Selenite F and Sodium tetrathionate broth (Harvey and Price, 1979). The choice of enrichment to be used to determined

by the specialist interest of the bacteriologist. For example Selenite F is known to be essential for the enrichment of Salmonella typhi primarily and other Salmonellae in faecel samples (Leifson, 1936; Harvey and Price, 1964). The same workers also demonstrated that Kauffman tetrathionate broth was more efficient as an enrichment medium for the isolation of Salmonella from contaminated food and water.

In the case of Shigella pre-enrichment of suspected material in Seleite F have been found to increase yield by 4.3 and 3.6 percent respectively (Sen, 1964; Bhat et al., 1971). Maximum number of isolations of both Shigella and Salmonella organism was also obtained by Rollender et al. (1969) after initial pre-enrichment in GN broth.

Various media have been developed for the isolation and identification of Salmonella and Shigella organisms from faecel samples. These include xylose-lysine-desoxycholate agar (XLD), eosine-methylene blue (EMB) Hektoen enteric agar (HEA), desoxycholate-citrate agar (DCA), Salmonellae-Shigellae agar (SSA) MacConkey

agar (MA), Brilliant green agar (BGA),and Bismuth sulphate agar (BSA) (Goyal et al., 1981). However many of the selective and differential plating media are infact so inhibitory as to prevent the growth of the very organism which they are were intended to promote (Rollender et al., 1969). SSA and BSA belong to this category (Bhat et al., 1971).

Since one medium is not sufficient for the isolation of enteric pathogens from faeces, Goyal et al.(1981) recommended a combination of MA and XLD or MA + HEA based on comparative studies using eight different media. However both HEA and XLD are known to be very expensive and are rare to comeby in Nigeria. Equally effective for the isolation of Shigella spp is the MacConkey-Tellurite medium (1 ug/ml potassium tellurite in MacConkey agar) which has been used in the isolation of both Vibrio cholerae and Shigella organisms (Sen, 1964).

Generally, the differentiation and characterization of the enteric bacilli is based upon antigenic studies. A useful primary differentiation is made on the basis of the lactose fermentation which

is roughly correlated with pathogenicity. The coliform bacteria ferments this sugar rapidly with the formation of acid and gas in twenty four hours while Shigellae and Salmonellae (essentially pathogens) do not ferment it. Similarly the dysentery bacilli or Shigellae divide into two groups on the basis of fermentations of mannitol and are anaerogenic (Ewing, 1958). The Salmonella group in general produced gaseous fermentations; the typhoid bacillus is typically anaerogenic.

A great variety of cultural and biochemical reactions including the conventional sugar fermentations, motility, formation of Indole from tryptophane and various types of specialized tests such as the utilization of tartaric acids and malonate, amino acid decarboxylase activity etc. have been useful in the physiological characterization of the Salmonella and Shigella groups (Edwards and Ewing, 1972).

Partial or complete identification of Shigella and Salmonella is usually carried out by the use of antisera containing appropriate antibodies.

The vast number of different Salmonella serotypes (over 2,000 are known) and the numerous antisera required for the recognition of all these (a total of 58 different "O" antigents and 72 "H"antigens) make Salmonella typing a "specialization" requiring a lot of technical skills and know-how.

A number of Salmonella species can further be subdivided into types on the basis of their susceptibility to distinct bacterophages (Garg and Singh, 1974). Since the types exhibit a high degree of stability under natural conditions, phage typing is of immense value in epidemiological investigations in tracing source of typhoid or paratyphoid fever.

In the case of Shigellae, phage typing is not very popular and serotyping is relatively easy. Based on somatic antigens four species of dysentery bacillus are recognized viz: S. dysenteriae, S. flexneri, S. boydii and S. sonnei. These are further divided into various serotypes.

In this study the detailed biochemical characterization of the Salmonella and Shigella isolates was carried out in order to differentiate the isolates from other enteric bacteria.

Serological characterization was also carried out in order to "pigeon-hole" these isolates into respective serotypes.

MATERIALS AND METHODS

Collection of samples and isolation of organisms:

A total of 10,000 faecal samples sent to the laboratory from patients with cases of provisionally diagnosed diarrhoea in three different hospitals in Ibadan, namely: The University College Hospital (6,000), State Hospitals at Adeoyo (3,000) and Ring Road (1,000) were screened for the presence of Salmonella and Shigella. 200 samples were also taken from diarrhoec piglets at the Teaching and Research Farm University of Ibadan. The later were collected by using sterile cotton wool swabs.

Cultivation techniques

A loopful of each stool sample (preferably from blood and mucoid area if any) was inoculated on plates of MacConkey and Deoxycholate citrate agar. Also bijou bottles containing 4ml Selenite F broth were similarly inoculated to enrich the isolation of any Shigella and Salmonella if present. All cultures were incubated at 37 C for 24 hours.

The Selenite F was later subcultured into fresh DCA and one MacConkey agar plates containing 1 ug/ml

potassium tellurite (MTA) and incubated at 37 C for another 24 hours. The plates were later read and non-lactose fermenting colonies were selected.

Purification of Isolates:

Cultures sharing predominantly non-lactose fermentation were subcultured on fresh DCA to ensure purity. Pure cultures were streaked onto nutrient agar slopes and kept on the shelve and later studied in detail.

Biochemical characterization of Isolates:

The biochemical characterization of the isolates was according to Edward and Ewing (1972) and Cowan and Steel (1979) as follows:

(i) Fermentation of Carbohydrate: The isolates were tested in batches to ensure better reproducibility. They were innoculated into fresh agar plates. A descrete pure colony of each isolate was inoculated into carbohydrate broth containing a Durham tube. The medium consists of nutrient broth plus 0.5% of the particular carbohydrate e.g. glucose, plus Andrade indicator. Since the end products of carbohydrate

fermentation are acid or acid and gas the indicator revealed production of acid and the inverted vial trapped the gases where produced. The carbohydrates employed for this test were: glucose, lactose, mannitol, xylose, sucrose, dulcitol, salicin, rhamnose, sorbitol, arabinose raffinose, adonitol, maltose and inositol.

Test for Hydrogen sulphide: With the straight wire loop one of the selected colonies was carefully picked and inoculated into a tube each of Kliger Iron Agar (KIA) by stabbling the but and streaking the slope. A test tube of peptone water as well as a plate of MacConkey agar plate were innoculated for purify. All the cultures were incubated at 37 C for 24 hours.

Hydrogen sulphide was produced by organisms capable of reducing Sulphur-bearing compounds such as Sodium thiosulphate present in the medium. The hydrogen sulphide reacted with iron salts in the medium to form a black precipitate of ferric sulphide. The presence of black colour along the inoculation channel only was taken as a positive result.

(iii) Test for Indole production: A twenty four hour broth culture of each of the isolate was used. About 1 ml of Kovacs reagent was delivered into the broth culture. The production of Indole from the metabolism of tryptophan by the bacterial enzyme typtophanase was detected by the development of a pink to red colour.

(iv) Cytochrome oxidase test: A piece of Whatman No. 2 filter paper in a Petridish was impregnated with a few drops of freshly prepared oxidase reagent (1.0% tetramethyl paraphenylene hydrochloride). A loopful of the growth of the isolate on a agar plate was smeared onto a small area of the reagent-impregnated paper. A positive reaction was denoted by a dark purple colour within 30 seconds. Positive and negative controls were set up using Pseudomonas aeruginosa and Escherichia coli which gave positive and negative reactions respectively for this test.

(v) Urea test: A slope of Christensen's urea agar was innoculated heavily with the isolate. It was incubated overnight and examined. The presence of enzyme urease, which hydrolysed urea to ammonia,

was detected by a change of colour of phenol red indicator in the medium from straw or yellow (acid) to pink or purple (alkaline). Pale pink colour was disregarded according to the manufactuer's instruction.

(vi) Citrate utilization test: Koser's citrate broth in a bijou bottle was innoculated with the isolate using a straight wire and later incubated and 30 C. The test detected those organisms which were capable of utilising citrate in the form of its sodium salt as the sole C source. Organism capable of utilising citrate produced alkaline metabolites which changed the pH of the medium, resulting in a change in colour of the bromothymol blue indicator from green (neutral) to deep-blue (alkaline).

(vii) Motility: Motility was performed by the conventional hanging-drop technique. Each isolate was grown in peptone water and incubated at 37 C overnight. One drop of the broth culture was placed in the centre of a cover-slip. A ring was made in the centre of a microscopic slide with plasticine.

The slide was inverted over the coverslip so that the ring encircled the culture. The slide was quickly turned so that the coverslip was then on top. The organism was examined for mobility using the X10 and X40 objectives.

Decarboxylase reaction: Other biochemical tests included lysine decarboxylase, lysine deaminase, ornithine decarboxlase; Arginine dihydrolase. For the decarboxylase reaction, tubes of the four media (arginine, lysine, ornithine and control) were inoculated through the paraffin layer with the aid of a straight wire. They were incubated and examined daily for four days. Formation of a violet colouration gave a positive decarboxylation reaction.

Methyl red (MR) reaction: Glucose phosphate (MR) medium was inoculated with each isolate and incubated at 37 C for 2 days. 2 drops of Methyl red solution was added, shaken and examined. Formation of a red/orange colouration proved a positive reaction.

Voges Proskauer reaction: After reading the MR reaction the same culture was employed for the VP test. O.6 ml 5% α-napIthol solution and 0.2ml 40% KOH was added. The tube was shaken and examined and 1 hr later, a strong red colour was observed in positive reaction.

KCN test: Bijou bottle containing KCN broth was inoculated with a loopful of an overnight broth culture of the isolate. The bottle cap was tightly screwed down and incubated for up to 48 h. at 37 C Turbidity (indicating growth) was observed in positive cultures.

Serological identification of Isolates:

Slide agglutination test was performed on each of the isolates as follows: A loopful of saline solution was placed on a clean glass slide. To this was added a small amount of the pure culture of the isolate to be identified and the later was thoroughly emulsified on the saline solution until it was faintly turbid. A loopful of the commercially prepared agglutinating sera (supplied by Wellcome Research Laboratories, England) was placed beside the bacterial emulsion

on the same slide. The two were later mixed using the inoculating loop. The slide was rocked gently back and forth to afford proper mixing of antiserum and bacteria. The mixture was observed against a white background in the presence of a good light source. Agglutination or clumping of cells indicated a positive reaction.

In case of Salmonella identification, Salmonella polyvalent "O" (A-G) serum and polyvalent A (Composite "phase 1 and phase 2") was employed.

The Shigellae were classified into Shigella dysenteriae (Group A), Shigella flexneri (Group B) and Shigella boydii (Group C) by using commercially prepared agglutinating sera obtained from Wellcome Research Laboratories, England.

RESULT

Of 10,200 stool specimens cultured, 31 isolates of Shigella and 22 isolates Salmonella were made.

Colonial Morphology and Staining: Shigella on DCA were round, pale, 2-3 millimeter diameter, slightly convex colonies with entire edge. When viewed against a dark background, they were whitish and slightly opaque. Salmonella appear like Shigella on D.C.A. The difference lies in the production of hydrogen sulphide in the medium. Both bacilli were gram negative rods closely resembling and indistinguishable from the coliform bacteria. No particular arrangement of the cells was apparent on microscopic examination. No capsule was apparent and spores were not formed. Table 3.1 (Appendix 4) shows the biochemical features of the organisms encountered in the study.

Carbohydrate fermentation: Organisms later identified as Shigella were found to produce acid but no gas in glucose, mannitol, arabinose and trehalose. Other carbohydrates including lactose were not attacked. The other non-lactose fermenters showing acid and gas production in glucose, mannitol, dulcitol,

sorbitol, arabinose, xylose, Rhamnose and Maltose were later identified as Salmonella typhi. In general the Salmonella were found to utilise more carbohydrate than Shigella.

Hydrogen sulphide production: All the Salmonella species were found to give a positive reaction in the Kliger's medium as indicated by the regular formation of black precipitate of ferric sulphide along the inoculation channel. The Shigella isolates did not produce hydrogen sulphide.

Indole production: The Salmonella isolates were found to be indole negative as well as the majority of the Shigella which were positive. In other words the Shigella organisms gave variable results to the indole test.

Oxidase production: All the isolates reacted negatively to the oxidase test. This shows that neither Salmonella nor Shigella produce cytochrome oxidase enzyme and hence failure to give a purple colouration when the oxidase reagent was applied to a smear of the culture isolates on filter paper.

Urease production: None of the isolate was found to be urea positive as there was no familiar cherry-red colour developing in any of the urea slants employed in the test.

Citrate utilization: Most of the Salmonella isolates (except those later identified as Salmonella typhi) were able to utilize citrate as a sole source of carbon whereas all the Shigella spp were citrate negative.

Motility: All the Salmonella tested in this study were found to be motile whereas all the Shigella isolates were non-motile.

Decarboxylase production: Organisms later identified to be Shigella failed to produce decarboxylase enzymes, hence arginine, lysine and ornithine were unaffected. All Salmonella isolates were positive for Arginine, lysine while some gave variable result when tested on ornithine.

KCN utilization: All strains failed to grow in the potassium cyanide broth after incubation for 48 hours.

MR and VP tests: The Salmonella isolates were positive for the methyl red and negative for Voges Proskauer reactions so also were the Shigella organisms.

Of the thirty-one Shigella isolates, twenty-four were serotyped as Shigella flexneri; four Shigella dysenteriae and three Shigella boydii. The Shigella flexneri were mainly type 6, 2, 3, 1 and 4 while S. dysenteriae were of types 2 and 3.

Nine isolates of Salmonella were identified as *Salmonella typhi*. The remaining isolates were classified as Salmonella species.

DISCUSSION

In this work MacConkey agar (MA), Desoxycholate-citrate agar (DCA) and MacConkey-Tellurite agar (MTA) were used for the isolation of Shigella and Salmonella present in faecal samples taken from diarrhoeic cases. The choice of these selective media was made on common sense ground. Xylose-lysine desoxycholate agar (XLD) and Hektoen enteric agar (HEA), both superior to the above three are known to be very expensive and very rare to come by in this country.

DCA has been shown to be superior to MA (Bhat et al., 1971) and is readily available. According to Rollender et al. (1969) the sodium-deoxycholate content allows for the inhibition of coliforms without decreasing the ability of the medium to support the growth of Salmonella and Shigella organisms. MacConkey agar contains bile salt which is also inhibitory to gram positive organisms and in combination with potassium-tellurite its selectivity is enhanced for the isolation of Shigella.

Typical non-lactose fermenters appears as colourness on both DCA and MacConkey. Shigella organisms appear as grey-centered colourless

colonies on Tellurite-MacConkey agar. However, identification of Salmonella and Shigella based on morphological appearance, is subjective. While both pathogens ordinally produce colonies of typical aspects on selective medium, it may be altered by growth in close association with other organisms. Also, sometimes these pathogens produce colonies of a typical appearance for reasons that are not entirely clear. Thus the differentiation of enteric pathogens based on their inability to ferment lactose may not be infallable because once a while lactose fermenting colonies of Salmonella do come up. Although not encountered in this study, the Shigella sonnei have always been recognized as late lactose fermenters.

This investigation established that Shigellae are the more frequent causative agents of diarrhoea in people whose stools were sampled. Some of the Shigellea were isolated on freshly prepared Desoxycholate citrate agar (DCA) after preliminary enrichment in selenite F broth. Previous work by Adetosoye and Rolitu (1986) found the isolation rate of Shigella in stool samples to be higher (42.1 perscent) when compared with that of E. coli (33.7 percent) and Salmonellae (24.6 percent) respectively.

In this study 60% percent of the isolates were Shigellae while Salmonellae acounted for 40 percent. Of the two hundred swab samples of diarrhoeic piglets processed only one grew Shigella while there was no Salmonella encountered. Shigella was previously thought to be a pathogen of man and primates only, but has since been isolated from non-human source (Formal et al., 1958). Nyaga et al. (1985) reported the isolation of S. dysenteriae from domestic fowl in Kenya. Ibu et al. (1987) also isolated S. flexneri from diarrhoeic piglets in Jos. It can be assumed that these organism were acquired from contaminated feeds.

Among the Shigella isolates identified S. flexneri was the most prevalent as twenty-four of the thirty-one isolates were so identified. This is in agreement with the work of Ogunbi (1971), Rahaman (1984), and Adetosoye and Rotilu (1986). In the experience of these workers S. flexneri ranked higest in the freguency of isolation among other Shigellae from clinical specimens. Mutanda et al. (1979) also reported that in East African countries, namely:

Kenya, Tanzania and Uganda, S. flexneri was the most frequently isolated species, followed by S. sonnei. S. boyii came third S. dsenteriae was least. Usually one particular species, dominates in a community or country . For example in the developed countries such as Britain, U.S.A and Japan, S. sonnei accounted for most of the cases of shigellosis (Roseberg et al., 1973). In this study late lactose fermenting S. sonnei colonies were not encountered throughout the investigation. Earlier report by Adetoyose and Rotilu (1986) also did not indicate the presence of S. sonnei in any of the stool samples screened. The failure to isolate S. sonnei may be due to the fact that the organism is prevalent only in the temperate countries.

Overall there seems to be low Shigella and Salmonella in suspected diarrhoea stools screened in this work. Odugbemi et. al., (1982) reported 3.3 percent Shigella and 0.9 percent Salmonella when six hundred and seventy four stool samples were examined in Lagos. Earlier, Ogunbiyi (1971) obtained 8.5 percent prevalence in the number of Shigella isolates. This

consistently low percentage of isolation may be attributed to the fact that there are numerous other etiological agents that cause diarrhoea apart from these two organisms. For example it is known that rotavirus is an important etiology of diarrhoea in children (Kumar et al., 1984) and well over 80 percent diarrhoea in infants have been attributed to Rotaviral infection. Escherichia coli has also been implicated in cases of serious diarrhoea afflicting man and animals (Adetosoye, 1980a). Another reason is that only limited number of piglets were sampled. In the course of this investigation many lactose-fermenting E. coli showed up repeatedly on the isolating plates but since they were not the primary aim of this research they were usually ignored and such plates discarded. Undoubted some of these E. coli might have been the agent of diarrhoea.

Twenty two Salmonellea were found in all the stool samples screened. This is not surprising because infection with Salmonella organisms is known to be widespread, and apart from clinical Salmonellosis,

both man and animals are known to be carriers to Salmonellae. Many published work on Salmonellae in Nigeria between 1956 and 1962 emanated from some medical expatriates working in the University College Ibadan, notably P. Collard; R, Sen; S. Lapage; D. Montefiore; R. Johnson and W. Plowright. Some of the Salmonella strains isolated were from patients suffering from various ailments other than typhoid. Of the two hundred and eighty two pathogens isolated from Veterinary Clinic of University of Ibadan between 1971 and 1975, Salmonella accounted for 17.3 percent (49 isolates) of the total number (Falade, Ojo and Ogunnariwo, 1977). In this work forty percent of the total number of isolates were found to comprise of S. typhi and other Salmonella species.

According to Collard and Sen (1957) the most predominant spe cies of Salmonella in Ibadan were S. typhi and S. agama. In this present work S. typhi accounted for forty one percent of the total number of Salmonela isolates. It thus confirms that cases of typhoid fever is still very prevalent in Ibadan. For example in India, Saxena et al. (1983) found S. typhimurium and S. typhi to be the most prevalent of all the Salmonella serotypes encountered.

The number of human lives claimed by Salmonella infection is staggering, Veterinary Salmonellosis is also a big threat to the animal industry. For example in Britain 14,351 human and 11,682 Veterinary infections were recorded between 1968 to 1980 (Edel et al., 1981), The age group at the highest risk were infants less than one year old and adults in the senior age bracket. Most of the human infections were known to occurs in the hospital on person-to-person spread. In animals, the usual source of infection is through contaminated feeds. The habit of promoting growth by feeding animals with antibiotics has led to the selection for resistant strains.

Persistant reports of isolation of Salmonellae and Shigellae in our environment calls for urgent attention by health authorities in this country. Although it may never be possible to eliminate Salmonella and Shigella oragnisms completely from our environment and farming set up, concerted efforts should be made to minimise the spread of these two deadly organisms by way of provision of good water supply. The current campaign on hygenic living should be encourages since "cleaniness is next to holyness. In the Veterinary practices a lot of controls need be introduced to curb the dissemination of these pathogens.

The low level of *Shigella* isolates encountered in piglets may be due to the fact that (i) Shigella is not normally a pathogen of animals.

(ii) The small sample size

(iii) The hygeine status of the environment in which the piglets were raised.

CHAPTER FOUR

INFECTIOUS DRUG RESISTANCE IN SHIGELLAE AND SALMONELLAE ISOLATED FROM DIARRHOEIC CASES

INTRODUCTION

Shigellosis and Salmonellosis are endemic in various parts of the world including Nigeria. The causative agents of these diseases are Shigella and Salomella spp. respectively.

In the past, the Sulphonamide drugs were the drug of choice for bacillary dysentery (Shigellosis) (Cheever, 1952). Other drugs which have been found useful include Tetracyclines, Ampicillin, Trimethoprim-sulphamethoxazole and Nalidixic acid (Panhotra et al., 1985).

In the case of Salmonella infections, chloramphenicol has been the most successful drug. The recommended dose in adult is 500mg every four hours (Mandal, 1979). Other drugs found useful in the treatment of Salmonellosis include Ampicillin (Roberston et al., 1986); Co-trimoxazole (Akinkugbe et al., 1986) Amoxycillin (Pillay, et al., 1975) and Furazolidone (Hezog, 1976).

resistant Salmonella typhi in Nigeria by Njoku-Obi and Njoku-Obi (1965), other workers have reported a wide-spread incidence of antibiotic resistance in enteric pathogens in this environment (Ojo, 1973; Adetosoye, 1980; Ibu et al., 1987; Olukoya et al., 1988b). Such resistant strains carry genes encoding products that inactivate those antibacterial agents; keep them from reaching their target sites or provide alternaives for those sites that have been blocked. Some of these antibacterial resistance genes are on the bacterial chromosome, but the majority are on extrachromosomal genetic elements called plasmids that may transfer themselves to other strains or species of bacteria (Jacoby and Swartz, 1980).

The original discovery of transmissible drug resistance was made by Japanese workers in 1959 (Ochiai, et al., 1959). The transmissible agents responsible for drug resistance are known as resistance factors or R factors (Mitsuhashi, 1960). Although the commonest transmissible resistance pattern was against Sm, TC, CM, and Su, combinations of resistance to four, three or two of these drugs and single drug resistance were known to be transferable (Datta, 1965).

Evidence that R factors are widespread comes from many sources and they have been found wherever they were looked for (Rangnekar et al., 1983; Adetosoye and Rotilu, 1986; Olukoya et al., 1988b).

In this work, the drug susceptibility patterns of Shigellae and Salmonellae isolated from diarrhoeic cases in Ibadan were investigated. The isolates were also screened for the presence of transmissible resistance plasmids. The later were identified and characterized in Chapter 5.

MATERIALS AND METHODS

Bacterial isolates:

Fifty two bacterial isolates from diarrhoeal cases were employed in this study. They comprised of 23 S. flexneri, 4 S. dysenteriae, 3 S. boydii. Others are 9 S. typhi and 13 Salmonella spp. The isolates were maintained on nutrient agar slants prior to use.

Antimicrobial susceptibility testing:

1. Disc diffusion method: The modified method of Bauer et al. (1966) was employed.

   Five colonies of each pure bacterial culture were inoculated into 5ml sterile nutrient broth (Oxoid) in a bijou bottle. The broth cultures were then incubated overnight at 37 C and diluted five times to give an approximate bacterial concentration of 10^6 cells per ml.(determined by Miles and Mistra method).

   The diluted broth culture was asceptically poured and spread on the whole surface of freshly prepared sensitivity test agar (Difeco). Surplus suspension was decanted from the surface of the agar plates which were allowed to dry at room temperature for 3 to 5 minutes. Later multo

discs Oxoid Code No. 3857E containing appropriate concentrations of the antibiotic substances (Appendix 1) were placed on the agar with sterile forceps. The disc were spaced far apart to prevent overlapping rings of inhibition and gently pressed down to ensure contact with the surface to the agar. For a series of tests, E. coli NCTC 10418 was used as sensitive control strain. Plates were incubated at 37 C for 24 hours.

After overnight incubation, the zones of diameters (including the 6mm discs) were measured with a ruler. A reading of 6mm indicated no zone of inhibition. The end-point was taken as complete inhibition of growth as determined by the naked eye. The diameters of zone of inhibition were compared with those of control strains. (See Appendix 5).

2. Minimal inhibitory concentration (MIC) Determination:

The MIC of each of the antibiotic was determined for each of the isolates in order to obviate the shortcomings of the traditional disc diffusion test which is prone to problems (Bailey et al., 1981.

Stock solutions of Chloramphenicol, ampicillin, streptomycin and tetracycline were respectively made in sterile distilled water. The concentration of the stock solutions was 2000ug/ml. Serial dilutions of each antibiotic was carried out in 10 tubes, the first of which contained 0.128 ml of the Stock solution of the antibiotic and 9.872 ml of nutrient broth to give a concentration of 128 ug/ml of the antibiotic. After thorough mixing 4.936 ml content of the first tube was asceptically transfered into a second tube and mixed with an equal volume of nutrient broth to give a concentration of 64 ug/ml. This procedure was repeated for the next eight tubes. Concentrations of the antibiotics in the tubes were 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 respectively. One millitre of 8 hours old nutrient broth cultue of each isolate was diluted with 4ml of nutrient broth. 1 ml of this diluted broth culture was innoculated into the tubes containing the serially diluted antibiotics above and incubated at 37 C for 24 hrs. E. coli 10418 was used as a control. The Minimal Inhibitory Concentration of the antibiotic under test is the lowest concentration of the antibiotic which inhibited the organism.

Coniugation (interrupted mating) experiments:

Plasmid transfer experiment was performed using the standard procedure. E. coli K 12 5 25 resistant to 200ug/ml nalidixic acid (received from Dr. A.I. Adetosoye) was used as recipient. The donors were the Shigella and Salmonella isolates which have been found by the disc diffusion method (above) to be resitant to ampicillin, tetracycline, streptomycin and chloramphenicol either signly or in combinations.

The donors and the recipients were grown separately in Brain Heart Infusion broth for four hours. Aliquot of 0.5ml of eah donor cell was mixed with 1ml of recipient cells in sterile Bijou bottles. To this was added 3.5mls of freshly prepared Brain Heart Infusion broth and incubated overnight for 18 hour at 37 C without shaking (Adetosoye and Rotilu, 1986).

Selection method:

The selection was carried out using freshly prepared MacConkey agar plates to which has been incorporated various antibiotics formulations (see Table 4.1 below).

Table 4.1

SELECTIVE MEDIA OF FIVE FORMULATIONS

Anti-biotic	AMP	TE	C	S	Na
Medium					
1	25				100
2		25			100
3			25		100
4				25	100
5					100

Key: Amp: Ampicillin,
Te: Tetracycline,
C: Chloramphenicol
S: Streptomycin,
Na: Nalidixic acid

Selection plates: On this medium only E.coli transconjugants that has acquired resistance to the drug(s) incorporated into the medium grew.

Test for Transconjugants:

After inoculating the selection plates with the mixed cultivation broth, the plates were incubated for 24 hours at 37 C. Colonies were picked from the selection medium and tested for antimicrobial susceptibility by disc diffusion method to establish the transfer of donor resistance determinant.

RESULTS

Zones of inhibition produced in a lawn of organism plated from diluted overnight broth culture were measured (Table 4.2a) (Appendix 5). The resistance patterns of the isolates to the antibiotic used is shown in Table 4.2b.

The resistance of the Shigellae isolates to each of the four major antibiotics were of the following order: Twenty eight of the isolates were resistant to Streptomycin, twenty seven to Tetracycline, twenty one to Ampicillin and twenty to Chloramphenicol.

The Salmonellae shared a marked reduction in number of resistant strains on the average: 19 strains were resistant to streptomycin, 8 to tetracycline, 8 to ampicillin and 8 to chloramphenicol. None of the 52 isolates was susceptible to all the antibiotics tested. Overall, twenty one drug resistance patterns were seen in the 52 isolates. However, 10 patterns accounted for 78% of the total (Table 4.2b). The isolates were resistant to minimum of one and maximum of seven antibiotics. The most common pattern was T-CT-F-A-S-C-Te (20%), followed by T-CT-F-A-S-Te (10%) while four other resistant patterns including CT-S scored 8% each.

The seven drug resistant pattern was more common among Shigella isolates (approx. 33%). Also pattern T-CT-S-Te-C and T-CT-A-S-Te-C both accounting for 16% were not encountered in the Salmonella isolates. On the other hand patterns CT-F-S and CT-S which occured prominently among Salmonella isolates were not encountered within the Shigella isolates.

Table 4.3a, b and c represent the comparative results of in vitro susceptibility tests on four commonly used antibiotics against the fifty-two isolates of Shigella and Salmonella. Ampicillin was found to be most active against Salmonella spp. The antibiotics inhibited all the isolates but one at a concentration of 64ug/ml. At 8ug/ml more than 40% of the isolates were inhibited (Table 4.3c). Streptmycin and tetracycline showed identical activites. At 32ug/ml almost all the Salmonella isolates were inhibited. The activity of chloramphenicol was slighly inferior to the three others. At concentration of 64ug/ml 3 of the isolates were found to be resistant. At concentrations greater than 128ug/ml one of the Salmonella isolates was not susceptible (Table 4.3a).

Overall, none of the antibiotics was found to be inhibitory at concentration lower than 1ug/ml.

The picture encountered in the Shigella isolates was significanly different. There was a consistently high resistancevalue throughout (Fig. 4.3a and b). The level of ampicillin resistance for Shigella was very high. Twenty-six of the isolates (87 percent) had MIC of >32ug/ml. At 128ug/ml twenty-seven percent of the isolates were uninhibited. The resistance of the Shigella isolates to the other antibiotics was found to be high. Twenty-One of the 31 Shigella isolates were not susceptible to 32 ug/ml of tetracycline. in vitro activity of streptomycin and chloramphenicol were similar as there were resistant Shigella organisms encountered at all the concentrations employed. For example, none of the isolate was inhibited at 4ug/ml of either antibiotic.

Table 4.4a shows the resistance patterns of some of the isolates to four commonly used antibiotics and the determinants transfered on mating each donor with E. coli K12 525, a lactose fermeter resistant to 200ug of Nalidixic acid used as the recipient.

All the forty-two isolates screened transfered ampicillin resistance (100 percent). Twenty-one isolates (50.0 percent) transfered two determinants, either A-Te or A-S or A-C. Seven (16.6 percent) transfered three determinants either A-Te-S or A-S-C. None of the isolates transfered four determinants. Among the Shigella species the chloramphenicol determinant was transfered at a low frequency as only three (10 percent) of the isolates were able to transfer the determinant as against six (42.8 percent) recorded in the Salmonella isolates. In all the cases where the resistance determinants were successfully transfered, the resultant transconjugants showed resistance to all the antibiotics whose determinants were transfered.

DISCUSSION

Antimicrobial susceptibility testing provides information by which therapy can be selected or modified by adjustment of the dosage of the antimicrobial agent already being administerd.

By the broth dilution method it was detected in this investigation that the minimal inhibitory concentration (MIC) of Ampicillin to the Salmonella species were generally fairly high. For example most of them were inhibited at concentrations between 4 to 128ug/ml (Table 4.3a and 4.3c). In an earlier work, Rotilu (1985) (Personal communication) found that all the Salmonella isolate he investigated were inhibited by ampicillin at a concentration of less than 30ug/ml. Generally, the level of resistance attained by a strain carrying R factor depends on the host cell and the resistance determinants (Anderson, 1968; Chopra et al., 1978). Thus there is variation from one bacterium to another. The higher MIC figures observed in this investigation when compared to Rotilu work may be due to the following reasons:

(i) increase in selective antibiotic pressure due to indiscriminate use

(ii) incomplete dosage

(iii) fake drugs that abound and

(iv) increasing self medication among the populace.

The Shigellae investigated in this work were generally more resistant than the Salmonellae (Table 4.3a and 4.3b) as significant number of them were not inhibited at antimicrobial concentration of 128ug/ml. Previous work in Ibadan by Rotilu (1985) (unpublished) showed that the Shigellae were highly resistant to many antibiotics, including ampicillin, streptomycin and chloramphenicol. Chun et al. (1984) described a streptomycin R factor confering resistance of more than 1,000ug/ml of the drug on a Shigella strain and to only 10ug/ml on a strain of E. coli. These workers pointed out that the same R factor usually produce different levels of resistance in different isolates.

It may be pointed out in passing however that the results of in vitro antimicrobial tests is usually at variance with the response of humans to antimicrobial therapy. Host factors remain the major determinants of the outcome of antimicrobial therapy (Weinstein and Dalton, 1968).

Such factors like adherence of bacteria to epithelial surfaces, susceptibility of bacteria to phagocytosis, chemotaxis of neutrophills and bactericidal activity of human serum play a significant role on the outcome of antibiotic therapy (Washington, 1979).

The fifty-three isolates (31 Shigella and 22 Salmonella) studied share twenty-one resistance patterns to eight antibiotics employed. Previous report by Adetosoye and Rotilu (1986) established nine patterns of resistance to six drugs by Salmonella, Shigella and E. coli isolates. In Ethiopia, Gebre-Yohannes and Habte-Gabr (1984) found nineteen drug resistance patterns in three hundred and sixty Shigella isolates. The multiple drug resistance problem occurs worldwide and is as result of indiscriminate use of antibiotics.

The subtherapeutic use of the antibiotics created indiscriminate selective pressure so that pathogens become resistant to a particular antibiotic or a related one and could transfer such resistance to other bacteria. In the opinion of many workers (Simmons and Stolley, 1974; O'Brien et al., 1987) many of such drugs need no longer be used.

Among the Shigella spp., S. flexneri has been associated with unusually high drug resistance. Gebre-Yohannes and Habte-Gabr (1984) had twelve resistant types on investigating one hundred and eighty-two S. flexneri isolates.

The practical use of multiple drug resistance survey is to guide treatment in areas or farms where laboratory facilities are unavailable (Noworyta, 1972), In such areas, information on drug resistance patterns, preferably linked to specific geographical areas, could be helpful in selecting appropriate drug therapy. Antibiotic resistance usually is unpredictable because patterns change abruptly. However, in Poland, Noworyta (1972) was able to delineate the "Western resistant" part of Poland from the "Eastern sensitive" part. It is doubtful if such a corollary can be made in this country because of constant intermingling of the populace and lack of large-scale systematic studies. However continuous surveillance of drug resistance in enteric pathogens (e.g. Shigella, Salmonella and E. coli) should be encourage in order to guide doctors in the choice of drugs. It should be emphasized, however, that antimicrobial therapy has not been a solution to the control of Salmonellosis and Shigellosis. This is mainly due to the problem of infections drug resistance.

[illegible] resistance.

It has been shown that members of the family Enterobacteriaceae including Salmonella and Shigella harboured R-factors which are transferable to sensitive recipients (Anderson, 1968; Adetosoye and Rotilu, 1986; Olukoya et al., 1988b). The autotransferability of R plasmids in this study was very high as all the isolates transferred single or multiple resistance determinants to E. coli K12 recipient. Many reports have indicated such high percentage of resistance transfer. Chun et al. (1984) recorded 73 percent transfer in Shigellae isolated in Korea. Rotilu (1985) (unpublished) reported 89.5 percent transfer in fifty-seven isolates of Salmonella, Shigella and E. coli from this environment (Ibadan area).

However, in vivo transfer of plasmid does not go on at this prolific rate due to the pH of the gut, oxidation-reduction potential, volatile acids, antagonistic metabolites in the gut and presence of other bacteria among other parameters (Watanabe, 1963). But one can safely assume that transfer is relatively efficient between genetically related bacteria.

The accepted model for the transfer of R-factor by conjugation suggested that the donor bacterium made a temporary contact with recipient bacterium by means of specialized hair-like appendages or pilli through which one of the replicated R-factor is transfered to the recipient cell while the other is retained by the donor (Anderson, 1965).

The molecular weights of These R-factors encountered in the work were later determined (Chapter 5).

TABLE 4.2B

PREVALENCE OF ANTIBIOTIC RESISTANCE IN SHIGELLA AND SALMONELLA IN FAECAL SAMPLES OF DIARRHOEIC PATIENTS

Pattern of Resistance		Salmonella	Shigella	Total	% of Total no of Isolate
T-CT-F-A-S-C-Te	(7)	1	10	11	20
T-CT-F-A-S-Te	(6)	2	3	5	10
T-CT-S-T-C	(5)	-	4	4	8
T-CT-A-S-T-C	(6)	-	4	4	8
CT-F-S	(3)	4	-	4	8
CT-S	(2)	4	-	4	8
T-CF-F-S-C-Te	(6)	1	1	2	4
CT	(1)	1	1	2	4
CT-F-A-S	4	2	-	2	4
T-CT-F-S-Te	5	-	1	1	2
CT-F-A-S-Te-C	6	-	1	1	2
T-F-S-Te-C	5	-	1	1	2
CT-F-S-Te	4	-	1	1	2
CT-F-S-Te-C	5	-	1	1	2
CT-S-C-Te	4	-	1	1	2
CT-F-A-S-C	5	-	1	1	2
T-CT-S-C-A-Te	6	1	-	1	2
CT-A-S	3	1	-	1	2
CT-S-C	3	1	-	1	2
CT-Te-S	3	2	-	2	4
Te	1	1	-	1	-

KEY: T = Trimetroprim Sulfamethoxazole;
CT = Colistin sulphate; F = Nitrofurantion;
S = Streptomycin; C = Chloramphenicol;
Te = Tetracycline; A = Ampicillin.

TABLE 4.3A

IN VITRO ACTIVITY OF ANTIBIOTICS AGAINST 30 SHIGELLA AND 22 SALMONELLA

Number of Isolates inhibited at different concentrations of the antibiotics ug/ml

AMPICILLIN

Isolates	1	2	4	8	16	32	64	128	> 128
Shigella	–	–	–	2	–	2	7	11	8
Salmonella	–	4	4	1	4	4	4	1	–

CHLORAMPHENICOL

Isolates	1	2	4	8	16	32	64	128	> 128
Shigella	–	–	–	1	2	5	9	3	10
Salmonella	–	–	5	6	4	1	3	1	1

STREPTOMCYIN

Isolates	1	2	4	8	16	32	64	128	> 128
Shigella	–	–	–	4	6	6	3	6	5
Salmonella	–	–	3	3	8	7	1	–	–

TETRACYCLINE

Isolates	1	2	4	8	16	32	64	128	> 128
Shigella	–	–	1	2	2	4	8	2	11
Salmonella	–	1	2	5	9	4	1	–	–

TABLE 4.3b

IN VITRO ACTIVITY OF ANTIBIOTICS AGAINST 30 Shigella Isolates

Organisms (No. of Isolates	Antibiotics	Percentages of isolates with MIC (ug/ml)								
		1	2	4	8	16	32	64	128	>128
Shigella spp. n = 30	Ampicillin	0	0	0	6.7	0	13.3	36.7	73.3	100
	Chloramphenicol	0	0	0	3.3	10.0	26.7	56.7	66.7	100
	Streptomycin	0	0	0	13.3	33.3	53.3	63.3	88.3	100
	Tetracycline	0	0	3.3	10.0	16.7	30.0	56.7	63.3	100

Table 4.3c

INVITRO ACTIVITY OF ANTIBIOTICS AGAINST 22 Salmonella ISOLATES

Organisms (No of Isolates)	Antibiotics	Percentage of isolates with MIC (ug/ml)								
		1	2	4	8	16	32	64	128	>128
Salmonella spp.	Ampicillin	0	18.2	36.4	40.9	59.1	77.3	95.5	100	0
	Chloramph-enicol	0	0	22.7	50.0	68.2	72.7	86.4	90.9	100
n = 22	Strepto-mycin	0	0	13.6	27.3	63.6	95.5	100	0	0
	Tetracy-cline	0	4.5	13.6	36.4	77.3	95.5	100	0	0

TABLE 4.4a

CONJUGATION: DETERMINANTS TRANSFERED ON MATING EACH DONOUR WITH E. COLI K_{12} Na^r

Isolate No.	Identification		Resistance phenotype of transconjugants			
			A 25	TE 25	S 25	C 25
2	S. flexneri	1	+	−	−	−
3	" "	6	+	−	−	−
4	" "	2	+	−	+	−
10	" "	2	+	−	−	+
11	" "	2	+	+	−	−
14	" "	2	+	−	−	−
16	" "	2	+	−	+	−
17	" "	2	+	−	+	−
18	" "	2	+	+	−	−
19	" "	2	+	+	+	−
20	" "	6	+	+	−	−
21	S. boydii		+	−	+	−
22	S. flexneri	6	+	−	−	−
24	" "	3	+	−	−	−
26	S. dysenteriae	3	+	+	+	−
27	" "		+	+	+	−
28	S. flexneri	2	+	+	−	−

Isolate No.	Identification		Resistance phenotype of tranconjugants			
			AMP 25	TE 25	S 25	C 25
29	S. dysenteriae	2	+	-	-	+
30	S. boydii		+	-	-	-
32	S. flexneri	2	+	-	-	+
34	" "	2	+	-	+	-
36	" "	2	+	+	+	-
37	" "	4	+	-	-	-
38	S. dysenteriae	2	+	-	-	-
44	S. flexneri	2	+	-	-	-
46	S. flexneri	3	+	-	-	-
48	S. flexneri	4	+	-	-	-
49	S. flexneri	6	+	-	-	-
50	S. typhi		+	-	-	-
51	" "		+	-	+	-
53	Salmonella spp.		+	+	-	-
54	" "		+	+	+	-
55	" "		+	-	-	+
56	S. typhi		+	-	+	+
57	Salmonella spp		+	-	-	-
58	" "		+	+	-	-

Isolate No.	Identification	Resistance phenotype of transconjugants			
		AMP 25	TE 25	S 25	C 25
59	Salmonella spp.	+	-	-	-
60	S. typhi	+	-	-	+
64	" "	+	-	-	-
65	Salmonella spp.	+	+	-	-
66	S. typhi	+	-	+	+
67	Salmonella	+	-	+	+

42 isolates were screened.

+ = growth of transconjugants on selection plate

- = no growth of transconjugants on selection plate

CHAPTER FIVE

ISOLATION AND CHARACTERIZATION OF PLASMIDS IN SHIGELLA AND SALMONELLA ISOLATED FROM DIARRHOEAL CASES

INTRODUCION

Plasmids are extrachromosomal genetic materials found virtually in all bacterial species (Anderson and Threlfal. 1974). Plasmids harboured by members of th Enterobacteriaceae mediate the transfer of a variety of genetic determinants, including those of drug resistance, haemolysin and enterotoxin synthesis, colicinogeny, heavy metal tolerance, resistance to ultraviolet irradiation, carbohydrate fermentation, hydrogen sulphide synthesis and other metabolic characters (Anderson and Threlfal, 1974). They have also been divided into thiry or more groups by method of compatibility characters (Jacoby and Swartz, 1980). Plasmids generally vary in size ranging from a few million to approximately 100 million daltons (Jacoby and Swartz, 1980). Those coding for resistance and virulence are known to be fairly large in size. The invasive ability of Shigella flexneri

has been shown to be due to possession of 140 megadalton plasmid (Sansonetti et al., 1982).

Plasmid profile analysis has been useful as an epidemiological tool in investigating outbreaks of enteric disease (Riley and Cohen, 1982). When used as a finger-print for a strain, the plasmid profile may aid in differentiation of strains or identifying the source of infection (Taylor et al., 1982). Such differentation may serve as a means of identifying related and unrelated Salmonella and Shigella. Thus in outbreaks in which there was a strong epidemiological association with a common exposure (e.g. a particular food or water) one would expect that Salmonella or Shigella which are resistant to many antibiotics would usually be the same genetically. However, plasmid profile analysis often proved this assumption to be false (Holmberg et al., 1984). It has also been demonstrated that plasmid profile analysis is as specific as phage typing in identifying related samples and either method is clearly superior to

antimicrobial susceptibility testing (Holmberg et al., 1984). The acquisition of new plasmid may also lead to complete change of phage type. A case in point was a change from type 204 to type 193 by S. typhimurim implicated in bovine salmonellosis in Britain (Willshaw et al., 1980). When first isolated in 1974 the organism was resistant to sulphonamide and tetracycline but later acquired two more plasmids which coded for resistance to five more antibiotics with concomitant change in phage type.

Over the years a number of methods have been evolved in the isolation and characterization of plasmids DNA. These methods involve three basic steps:

(i) Growth of the bacteria in a suitable medium;

(ii) Amplification of the plasmid;

(iii) Harvesting and lysis of the bacteria and purification of the plasmid DNA.

The general approach is to remove enzymatically or physically any rigid cell wall and then to lyse the cell with a detergent usually SDS (Sodium deodysyl sulphate). Nucleases are inactivated and the DNA is then recovered by ethanol precipitation.

Purification methods involve preferential removal of long strands of DNA from the covalently closed circular strands of DNA. Determination of the plasmid DNA molecular weight is usually done by use of the polyacrilamide gel electrophoresis followed by staining with ethidium bromide (Birnboin and Dolly, 1979).

In this country there is little information on plasmid profile analysis of Shigellae and Salmonellae, associated with diarrhoea. Thus the aim of this present study is to:

(i) isolate and characterize the plasmid content of these organisms with a view to comparing them with those encountered elsewhere.

(ii) identify and characterize the antibiotic resistance (R) plasmids present in transconjugants obtained in Chapter 4.

MATERIALS AND METHODS

Bacterial isolates:

All the 53 bacterial isolates obtained from diarrhoeal cases were employed in this study. They comprised of 24 Shigella flexneri, 4. S.dysenteriae, 3. S. boydii, 9 S.typhi and 13 Salmonella spp. (Table 3.1). One of the isolates (S. flexneri) was obtained from diarrhoeic piglet. For the characterization of R plasmids, eleven of the transconjugants obtained in Chapter 4 were employed. These were carefully selected so that each of resistant determinant successfully transfered was represented (Table 5.2). An E. coli strain (V517) containing multilple plasmid obtained from Dr. Olukoya of Dept. of Genetics and Oncology, Nigeria Institute for Medical Research, Yaba, was used as size reference.

Plasmid DNA isolation procedure: This was carried out by the method of Birnboin and Dolly (1979)., but with certain modifications. Since the foremost objectives was to screen for the presence of plasmids, certain procedures seemed unnecessary. Treatment with RNase was omitted, since the concentration of NaOH used was

sufficient to remove RNA. As the phenol required re-distillation, chloroform alone was used instead of the phenol-chloroform mixture.

Bacteria were grown overnight in Trioticase Soy Broth (TSB). One and a half millilitres of the culture was poured into an Eppendorf tube and centrifuged at 8,000 x g for 2 mins. The medium was removed by aspiration and the bacterial pellet was resuspended in 100 ul of an ice-cold solution of 50mm glucose, 10mm Tris (ph 8.0). The mixture was held for 5 min. at room temperature. Then 200 ul of freshly prepared 0.25N NaOH, 1% sodium dodecyl sulphate (SDS) were added respectively. The tube was closed and the contents were mixed by inverting the tube rapidly two or three times. The tube was held on ice for 5 min. after which 150 ul of an ice-cold solution of potassium acetate (pH 4.8) was added. The tube was held on ice for 5 min. and then centrifuged for an additional 5 min. at 4 C. The supernatant was transfered to a clean tube and an equal volume of chloroform was added. The contents were mixed on vortex mixer and then centrifuged for 2 min. The supernatant was transfered to a new tube and two volumes of 95% ethanol were added to precipitate the DNA.

After mixing on a vortex mixer the tube was held at room temperature for 2 min. The tube was then centrifuged for 5 min. at room temperature and then the supernatant was removed. The liquid in the tube was allowed to drain away by placing it in an inverted position on a paper towel. One millilitre of 70% ethanol was added after vortex. Mixing again briefly, the tube was centrifuged for 5 min. The supernatant was removed and the tube was inverted to allow the pellet to dry. The pellet was resuspended in 50 ul of TE buffer (pH 8.0).

Agarose gel electrophoresis of DNA:

The procedure described by Meyers et.al (1976) was modified as follows:

0.7% agarose (supplied by Pharmacia, Sweden) was dissolve in Trisborate buffer (8.9mM) Tris base, 2.5MM disodium EDTA, and 8.9mM boric acid. A dye solution consisting of bromocresol blue (0.07%), SDS (7%) and glycerol (33%) in water was added at 5 ul per sample to the DNA samples prior to electrophoresis. Electrophoresis was carried out in vertical lucite slab gel apparatus. The dimension of the gel were 9.6 by 14.2 by 0.6cm.

Sample wells were made by use of lucite comb with 14 teeth, each 0.508cm wide and spaced by 0.478cm. The power source was a Heathkit regulated high voltage power supply, model IP-17, and electrophoresis was carried out at 60mA, 120V, for 2h or until the dye neared the bottom of the gel. The gel was then strained in a solution of ethidium bromide in water (0.4ug/ml) for 15 min. Direct visualization of the DNA bands without removal of gels from glass tubes was made possible by illuminating the gels with a long wavelenght u. v. light (Black-Ray B-100A, ultraviolet products, Inc.). The gels were later removed from the glass tubes, illuminated with a short-wave mineral light (uvs-54, ultraviolet Products Inc., Calif.) and photographed using type 57 film (ASA 3000) with a polaroid mp-3 hand camera equiped with Wratten K2 and 25 filters.

RESULT

Table 5.1 shows for each isolate, the number and size of different plasmids. It is seen from the table that most of the isolates contained more than one plasmid. Some of the Shigella isolates contained between eight and ten plasmids. One of the Salmonella isolates (No. 9) harboured nine plasmids while others contained between two and six plasmids per isolate.

Altogether, a total of one hundred and thirty plasmids were isolated from twenty-nine organisms and they belong to forty distinct types. By using plasmids of known molecular weights, the molecular weights of the plasmids isolated from the bacteria were determined to be within the range of 2.0×10^6 D 55.5×10^6 D most of them however are less than 10 Daltons. Figure 5.1 (a & b) show the agarose gel electrophoresis of R plasmids from the transconjugants. It revealed the presence of R plasmid as well as one to five other plasmids gained during conjugation (Table 5.2). The R plasmids range in size between 2.2 - 38 Mdal. The ampicillin resistance determinant (which all the isolate transfered) was carried on plasmid with

molecular weight of 13.3 Mdal. Others are streptomycin 36, tetracycline 38 and chloramphenicol 4.0Mdal. The isolates showing triple resistance patterns carry plasmids raging in size between 2.2-13.3 Mdal.

DISCUSSION

The agarose gel elecctrophoresis method for detecting covalently closed circular plasmid DNA and the estimation of plasmid mass is sensitive and does not require radio isotopes or ultracentrifugation. The method is simple and it employs readily available reagents. In practice, ordinary bench centrifuge can be used for spinning to separate the cell wall materials for DNA, A "black and white" ordinary camera and millimetre rule was used to record results. The method has been applied in the analysis of plasmid components of clinical isolates of E. coli, K. pneumonia, C. freundi and a host of other bacteria (Meyers et. al, 1976 and Olukoya et. al. 1988 a, b).

In the present study it was found to be effective in carrying out the plasmid profile survey of all the Salmonella and Shigella isolates screened. The migration of covalently closed circular (CCC) plasmid DNA were related to the molecular weights and single brands of DNA were observed for each plasmid specie (Fig. 5.1 a & b). The molecular weights were found to range between 2.0 and 55.5 x 10^6 daltons. Most of the plasmids have their molecular weights below 20 megadaltons (Mdal).

The data presented in Table 5.1 also indicated that nearly all the Shigella isolates possess extremely small plasmids ranging from 2.0 - 3.0. Mdal in size. Tacket et al. (1984) investigated the plasmid profiles of 136 Shigella isolates twelve of which contain only small cryptic plasmids of low molecular weight. Many of the isolates investigated in this work contain a number of plasmids which were common indicating that few Shigella clones account for Shigellosis in this country. This is in contrast to the work of Tacket et. al (1984) who demonstrated that Shigellosis in Bangladesh patients was caused by large number of Shigella clones possessing a variety of distinct plasmids. It was also found in this work that the plasmid profile of isolate number 2 (S. flexneri) recovered from piglet was identical with those isolated from human sources. This finding is in agreement with the work of Wilshaw et al. (1980) who demonstrated similar plasmid profile in epidemic strains S. typhimurium isolated from man and animal sources. The phenomeon suggests that the Shigella isolated from diarrhoeic piglet must have been contacted from handlers.

Consistent absence of very large molecular weight plasmids (>55.5kb) in all the Shigella and Salmonella isolates was noted in this work. Similar studies by Olukoya et.al (1988a) failed to demonstrate appreciable number of large molecular weight plasmids in most of the strains Of enteric pathogens isolated in Lagos. However, the large plasmids have been associated with the enteroinvasive Shigella (Kopecho et al., 1980; Sansonetti et al., 1982; Tacket et al., 1984). In an earlier investigation using Sereny test (Sereny, 1957) four out of the fourteen Shigella isolates screened were found to be invasive. They caused purulent keractoconjunctivities in guinea pig models, however subsequent plasmid profile studies fails to reveal any large plasmid in these invasive isolates. [illegible]. Two reasons can be adduced for this: either the isolates do not harbour these large plasmids or they have been lost during storage. It is known that loss of Shigella invasineness occur in storage in association with loss of large molecular weight plasmids (Kopecho et al., 1980: Sansonetti et al., 1982). The isolates

employed in this work were stored for lenght of time varying between 6 months to 2 years. It could be assumed that they might have lost their large molecular weight plasmids.

Eight of the nine Salmonella isolates screened contained between two and six plasmids. The only exception was isolate number 9 with as many as nine plasmids. The isolate might have acquired the extra plasmids promiscously from other bacteria in in the environment. Acquistion of new plasmids have been demonstrated in S. typhimurium (Willshaw et al., 1980) leading to resistance to more antibiotics.

Two of the Salmonella isolates (65 and 67) carry three identical plasmids and hence are genetically related. This is suggestive of a common source of infection in both patients from whom these isolates were obtained.

When compared with plasmids isolated from America by Holmberg et al. (1984) and Tacket et al. (1984) there is a marked difference in the molecular weights of the plasmid DNA contents of the Salmonella and Shigella isolates. Those encountered in this work are of smaller size (ranging from 2.0 to 55.5

Mdal) whereas those in America are larger (ranging from 2.0 to 200Mdal. This difference may be due to the fact that different strains are responsible for enteric diseases in different communities. This also emphasizes the value of plasmid profile studies as an epidemiological tool in investigating outbreaks of diarrhoea.

The sizes of resistance (R) plasmids detected in this work were also found to be small and vary between 2.2 and 13.3Mdal. The exception are tetracycline and streptomycin R plasmids (Molecular weights 36 and 38Mdal respectively). Previous work by Olukoya et al. (1988b) indicated a range of between 3.3 to 6.5Mdal for most determinats and 24Mdal for tetracycline resistance. Ana-Vincete and Da Almeida (1984) also reported the presence of 36 Mdal R plasmid in Salmonella agona isolated in Rio-de-Janeiro.
It thus appear that most R plasmids vary in size. Many are of small molecular weights while others are fairly large in size.

Generally, plasmids confer resistance to many antibiotics by a variety of mechanisms including coding for enzymes which modify or destroy such antibiotics.

TABLE 5.1

Number and size of different plasmids among Shigellae and Salmonella Isolates

Isolate Number	Identification		No. of plasmids	Distance move on gell (mm)	Size of plasmids (Mdal)
1	Shigella flexneri	3	2	25	12.0
				69	2.0
2	Shigella flexneri	1	8	25	12.0
				30	5.0
				43	4.0
				53	2.8
				59	2.5
				63	2.4
				69	2.2
				78	2.0
3	Shigella flexneri	6	8	24	12.0
				30	8.5
				40	5.3
				43	4.0
				53	2.8
				55	2.5
				68	2.2
4	Shigella flexneri	2	8	24	12.0
				30	8.5
				32	7.5
				34	6.8
				40	5.3
				43	4.0
				54	2.8

				61	2.5
5	S. dysenteriae	1		14	25.0
			9	22	14.0
				25	12.0
				31	8.0
				35	5.7
				41	5.0
				56	3.0
				61	2.5
				64	2.4
6	S. boydii			14	25.0
			10	21	15.0
				24	12.0
				27	10.0
				30	8.5
				34	6.8
				41	5.0
				55	3.0
				64	2.4
				67	2.3
				7	44.0
7	S. flexneri	3	8	12	29.0
				24	12.0
				32	7.5
				55	3.0
				64	3.4
				71	2.2
				76	2.0
19	S. flexneri	2	2	75	2.0
				80	2.0

20	S. flexneri	6	4	23	13.0
				61	2.5
				71	2.2
				78	2.0
21	S. boydii		3	25	12.0
				35	6.5
				60	2.5
22	S. flexneri 6		3	22	14.0
				73	2.0
				80	2.0
26	S. dysenteriae	3	5	7	4.40
				12	29.0
				62	2.5
				72	2.1
				76	2.0
27	S. dysenteriae	1	2	47	3.5
				52	3.0
28	S. flexneri	2	1	53	3.0
30	S. boydii		4	30	8.5
				32	7.5
				40	5.0
				51	3.1
32	S. flexneri	2	6	10	34.0
				25	12.0
				30	8.5
				41	5.2
				61	2.5
				70	2.2

33	S. flexneri	2	3	25	12.0
				61	2.5
				70	2.2
34	S. flexneri	2	1	53	3.0
36	S. flexneri	2	2	57	2.6
				63	2.5
37	S. flexneri	4	1	38	5.5
49	S. flexneri	6	7	19	17.0
				25	12.0
				35	6.5
				49	3.0
				58	2.7
				67	2.4
				72	2.2
8	Salmonella spp		2	25	12.0
				41	5.2
9	Salmonella spp		9	11	31.0
				21	15.0
				26	12.0
				30	8.5
				31	8.5
				42	4.5
				44	4.4
				74	2.0
				79	2.0
11	Salmonella spp		2	13	26
				24	12.5

55	Salmonella spp	5	30	8.5
			33	7.5
			40	5.0
			52	3.1
			58	2.4
61	S. typhi	4	5	55.5
			30	8.5
			60	2.5
			70	2.2
65	Salmonella spp	6	22	14.0
			24	12.5
			35	6.5
			40	5.0
			55	3.0
			60	2.5
66	S. typhi	1	5	55.5
			5	55.5
			23	14.0
67	Salmonella spp	4	24	12.5
			35	6.5

See standard curved (Appendix 3).

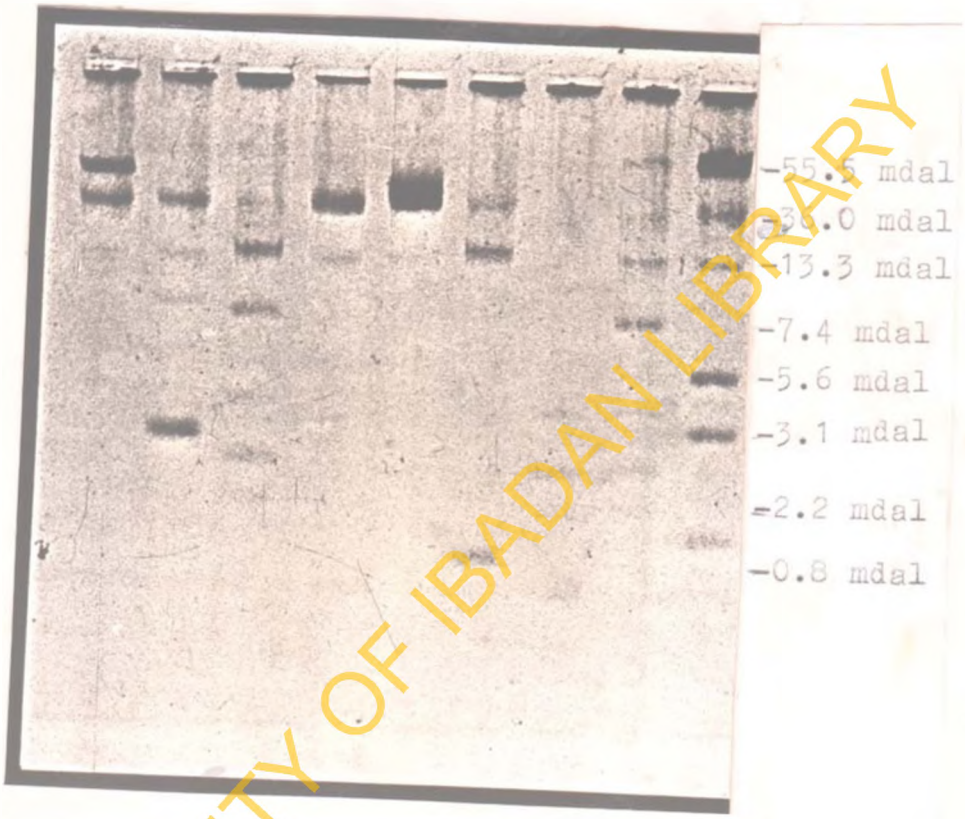


Fig.5. Agarose gel electrophoreseis of R. plasmids (and other) transfered to E. coli K_{12} Na^r during conjugation. From the left, column 1-2, Amp; columns 3-4. Amp and Strep, columns 5-6, Amp and Tet, columns 7-8, Amp and Chl (See also Table 5.2) Column 9 E. Coli plasmids of known sizes (standard).

Fig. 5.1b Agarose gel electrophoresis of R plasmids (and others) transfered to E. coli K12 Na r during conjugation. From left column 1 E. coli containing plasmids of known size (standard) column 2-3, Amp-Strep-Tet, and column 4 Amp-Step-chl (See also Table 5.2)

TABLE 5.2

PLASMID PROFILE OF TRANSCONJUGANTS

Column on agarose gell	Plasmids (Mdal)	Resistance	R plasmid
1	13.3, 36, 40	AMP	13.3
2	3.1, 7.4, 13.3, 36	Amp	13.3
3	2.8, 5.2, 7.4, 13.3, 36	Amp + Strep	13.3, 36
4	13.3, 36	Amp + Strep	13.3, 36
5	13.3, 38	Amp + tet	13.3, 38
6	13.3, 38	Amp + tet	13.3, 38
7	0.8, 3.1, 4.0	Amp + Chl	3.1, 40
8	3.1, 4.0, 7.4, 13.3, 36, 55.5	Amp + Chl	13.3, 3.1, 40
9	0.8, 1.0, 3.3, 5.6, 13.1 36, 55.5	Standard	-
10	2.2, 3.1, 4.5, 13.3	Amp + Strept tet	13.3, 2.2, 3.1
11	2.2, 3.1, 5.6, 13.3	Amp + Strep + Tet	13.3, 2.2, 3.1
12	1.0, 2.2, 3.1, 5.6	Amp+Strep + Chl	2.2, 3.1

CHAPTER SIX

VIRULENCE OF SHIGELLA SPECIES ISOLATED FROM CLINICAL DIARRHOIEC CASES

INTRODUCTION

Shigella is a gram negative bacterium in the family Enterobacteriacea. It causes a dysentery-like disease and dairrhoea referred to as Shigellosis. (Topley and Wilson, 1983). Shigellosis appears to be a two-organ disease; a jejunal fluid secretion caused by enterotoxins (Formal et al., 1983) and acute bacterial colitis resulting in dysentery (Keusch, 1978).

The enterotoxin of Shigella was first described in 1903 and purified in culture filterate of Sh. dysenteriae (Keusch et. al 1972a), S. flexneri and S. sonnei (Keusch and Jacewicz., 1977). The toxin was found to cause histological alterations in the colonic mucosa as well as transudation of fluid in the rabbit illeum (Keusch et al., 1972b). Its mechanism of action was found to be different from that of Vibrio cholerae and Escherichia coli in that it does not produce any marked increase in adenylate cyclase or

cyclic AMP (Flores et al., 1974 Steinberg et al.,1975). It does not cause depletion of goblet cell mucus (Steinberg et al., 1975). However, like E. coli and V. cholerae enterotoxins, intestinal receptors have also been demonstrated for Shigella enterotoxins in rabbits (Bresson et. al, 1984; Fuch et al., 1986; Mobassaleh et al., 1988).

The pathogesesis of Shigella infection has been studied in other animal models. Starvation, opiates and streptomycin pretreatments have been used singly and in combination to compensate for the natural resistance of animals such as guinea-pigs, rats and mice to challenges with Shigellae (Grady and Keusch, 1971). However in the highly manipulated animals, the infection often differs from the usual clinical pattern of human disease and knowledge gained from such studies have been subject of controversy (Keusch et al., 1976).

Rhesus monkeys (Branham et al., 1949) and human volunteers (DuPont et al., 1970; Levine et al., 1973) have also been used in the investiagation of the virulence of particular strains of Shigella. It is known that Shigella possess two potential pathogenic

modes of action. These are invasiveness and entero-toxigenticity. Levine et al. (1973) established that the primary virulence determinant of Shigella is the invasive capacity of the organism as strains which cannot penetrate and multiple within the colonic epithelial cells do not cause disease in humans.

Among the procedures available for testing the ability of Shigellae to penetrate epithelial cells and cause ulcerative lessions, is the Sereny test. This measures the capacity of the organism to cause kerato-conjunctivitis (Sereny, 1957). Invasion of Hela cell monolayers has also been used as a practical assay method (LaBrec, et. al., 1964).

The rabbit Illeal loop model discovered by De and Chatterjee (1953) has been used extensively to assay for heat-labile and heat-stable enterotoxins produced by bacteria associated with diarrhoea (Arms et al., 1965; Moon et al. 1970; Fuch et al., 1986). Apart from being an in vivo assay method, the ileal loop model affords the opportunity to measure directly the amount of fluid secreted by the rabbit small intestine upon expoture to enterotoxins.

These investigations were carried out to study the virulence of Shigella species isolated from cases of diarrhoea. Attempts were also made at inducing diarrhoea syndrome using pretreated laboratory animals as models.

MATERIALS AND METHODS

Test for invasiness - Sereny test:

Bacterial strains: Fourteen isolates of Shigella recovered from cases of diarrhoea were used. The isolates were maintained on nutrient agar slants prior to use. Broth cultures containing approximate 6×10^8 Shigella/ml (determined by the Miles and Mistra method) was prepared by growing the organism in nutrient broth and incubating overnight.

With the aid of sterile Pasteur pippete one drop of the suspension was introduced to the right eye of individual guinea pig. The left eye served as conrol. Each guinea pig was kept in separate cage and examined daily for four days for evidence of eye infection characterized by swelling, eye-ball depression, accummulation of fluid, mucus or blood and corneal opacity. The isolates which produced corneal opacity were later used to induce Shigellosis by oral administration on pretreated guinea pigs and mice.

Experimental Shigellosis in Guinea pigs and mice:

Bacterial strains: The organisms used in the study were those which produced keratoconjunctivitis in guinea pigs (Sereny test positive). They included

isolates No. 1 (S. flexneri 3), 3(S. flexneri 6). 5 (S. dysenteriae), and 7 (S. boydii). The isolates were kept on nutrient agar slants prior to use.

Pre-treatment of animals for experiment: The animals employed were grouped into three categories according to their pre-experimental treatments.

Group 1 consisted of 5 adult guinea pigs which were not treated;

Group 2 consisted of starved mice (deprived of food but given water ad. lib.).

Group 3 consisted 5 starved and calcium carbonate treated mice (fed with 25 mg $CaCo_3$ suspended in 1ml. distilled water to neutralize stomach acidity prior to the administration of bacterial culture) (Formal et al., 1958).

The three groups of animals were then fed with 1ml. of 24 hr old nutrient broth culture containing 30×10^{-8} ml of the isolates as determined by Miles and Mistra method. One animal in each group was not fed and thus served as the control.

Anal temperature of each of the treated animal was taken daily for seven days. Faecal samples of each animal was also examined. Failure of the animal to develop diarrhoeic symptoms (rise in body temperature as well as watery or bloody mucoid stool) within 72 hours was taken as a negative pathogenicity test.

Assay for enterotoxigenicity:

Bacterial strains: Twenty nine strains of Shigella were employed in this assay. The strains were those isolated from individual diarrhoeic case. They comprised of six serotypes of S.dysenteriae, twenty of S. flexneri and three of S. boydii. The strains were maintained on nutrient agar slants, kept at room temperature prior to use.

Preparation of crude bacterial enterotoxin: A loopful of each bacterial strain was subcultured on nutrient agar and five colonies were inoculated into 20ml of Tryptone Soy Broth and incubated at 37 C for 24 hrs. The broth culture was centrifuged at 18,000 rpm for 30 mins. at 10 C. The filterate was divided into two portions - one portion was heated at 65 C for 30 mins. while the other was left unheated. Both portions were kept at - 20 C until needed.

The control organisms were V. cholera:

(i) Ogawa 19339;

(ii) Ogawa 21140;

(iii) Classical 154; and

(iv) Escherichia coli 36004.

Animals: Five Newzealand rabbits weighing between 1.5 and 2.1 kg were used. They were deprived of food but were allowed to drink water ad lib. twenty four hours prior to surgery.

Surgical procedure: The rabbit was anaesthesized for surgery by injecting pentabarbitol through the ear vein to effect. The technique of preparing intestinal segments was similar to that described by Moon et al. (1974). All little incission of about 8cm long was made on the linea alba. The small intestine was carefully brought out and the duodenum was identified. About 10ml of warm sodium chloride solution was injected into the lumen of the illeum and squeezed through into the caecum. Ligatures were made with catgut carefully so as to avoid desruption of mesenteric capillaries. The intestine was ligated to form series of approximately 10cm segments

interrupted by 5cm sutures. An average of ten ligated illeal loops were made in each rabbit (Figs. 6.2 and 6.3).

Assay for heat labile (LT) enterotoxin: With the aid of a syringe, 1ml of the unheated (heat labile, LT) toxin was injected into each of the alternate 10cm loops. The interrupted 5ml loops were kept uninoculated. The first and last ligated intestinal loops contained the control, (LT) toxins i.e. V.cholera Ogawa 19339. The intestine was later replaced into the abdominal cavity and the incission closed into two layers by continuous suture.

The rabbit was kept in a warm place for 18 hours. Later it was killed by injecting pentabarbitol. The incission was opened and the intestine brought out. The results was recorded as follows:

Fluid accummulation evaluation: The volume of fluid in each ligted loop was measured in millilitres using a graduated syringe. The length of the loop was measure in cm with a ruler. A ratio of $\frac{\text{Volume}}{\text{Length}}$ was determined and values of 1 and above was regarded as positive LT.

Assay for heat stable (ST) enterotoxin: Fresh rabbits were used and the surgical procedure described above was followed. One millilitre of preheated enterotoxin (held at 65 C for 30 mins.) was injected into each of the larger (10cm) loops while the interrupted 5cm loops were kept uninoculated.

Escherichia coli 36004 enterotoxin was employed as positIve control. This was injected into the first loop. One millilitre of Tripticase Soy Broth was inoculated into the last loop. The operated rabbits were returned to their cages and killed 18 hours later by injecting pentabarbitol.

Fluid accummulation evaluation: The volume of fluid in each ligated loop was similarly measured in millilitres by using a syringe. The length of each loop was measured in cm. A ration Volume was determined and Length

value of 0.4 or above was regarded as positive for ST.

Histopathological evaluation: Appropriate segments of small intestine were carefully excised from rabbits during postmortem. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 5u. Sections were stained with Mayer's hematoxylin stain with 1% alcohol eosion as a counter stain and by the Brown and Brenn modification of the Gram stain for bacteria in tissue. The gross appearance of the

RESULT

Table 6.1 shows the result of invasive capabilities of fourteen *Shigella* isolates employed in the Sereny test. Four of the isolates (Nos. 1,3,5,7) were found to be invasive in that they induced mucopurulent discharge from the cornea of the infected animals (Fig. 6.1). In three of the four cases the effect became visible after 48 hours, first as reddening of the eyes. This later developed subsequently into inflamed purulent Keratoconjuctivitis. It is of interest to note that the four isolates comprised of two *S. flexneri*; one *S. dysenteriae* and one *S. boydii*. The remaining ten isolates were found to be negative since they produced no noticeable change in the cornea of the guinea pigs 96 hours after inoculation.

Experimental Shigellosis in Guinea pigs and Mice:

The four invasive *Shigella* isolates failed to induce diarrhoea when fed orally into pretreated guinea pigs and mice. A case of mucoid stool was encountered in one of the pretreated mice but on culturing the stool on MacConkey, it failed to grow *Shigella*. There was also no appreciable change in the anal temperature of the animals.

Assay for Enterotoxigenicity (ileal loop ligation test):

Introduction of enterotoxins of Shigella into the lumen of the ligated segments of small intestine in living rabbits frequently resulted in an accumulation of exudates to a varying degree causing dramatic dilation of the segments (Tables 6.2 and 6.3; Figs. 6.2 and 6.3).

In the assay for heat labile enterotoxin, six of the twenty-nine isolates tested caused fluid transudation and elicited positive response in segments of the rabbit small intestine. These were four S. flexneri, one S. dysenteriae and one S. boyii. The remaining twenty-four isolates only caused slight increased in the intestinal fluid volume in varying proportions (Table 6.2; and Figs. 6.2 and 6.3).

In the tests involving the heat stable enterotoxins four out of the eight isolates randomly selected recorded positive responses (Table 6.3). The four isolates comprises of three S. flexneri and one S.boydii.

One isolate, Shigella boydii (No. 30) was found to produce heat labile and heat stable enterotoxins respectively (Table 6.2 and 6.3).

Prominent histological changes were noticed in all the segments studied. This generally revealed haemorrhage, hyperaemia, generalized necrosis of the mucosa, cellular infilteration and oedema.

Histopathology:

V. cholerae +ve control 19339:

Section of small intestined revealed degeneration of the mucosa and moderate amount of neutrophilic infilterations.

S. flexneri 6 (+ve for Sereny and +ve Enterotoxin)

The sections of the small intestine revealed mild glandular degeneration with moderate lymphocytic infilterates.

S. dysenteria +ve for fluid accumulation (enterotoxigenic)

This section showed submucosal oedema and haemorrhage. There is also mild neutrophilic infilterate in the submucosa.

S. boydii +ve for fluid accumulation (Enterotoxigenic):

This section of the intestine shows degeneration of the mucosa, submucosal, oedema and neutrophilic infiltrations.

DISCUSSION

In previous investigations conducted by Silva et al. 1982 involving fifty eight strains of *Shigella*, twenty seven of them were able to induce keratoconjunctivits while the remaining thirty one were negative in the test. The relatively large number of avirulent isolates (Sereny negative) encountered in this study may be due to the fact the isolates have been maintained on slants for a fairly long period (over six months) before being tested. Thus, they may have lost the plasmids encoding for invasineness. Recent evidences have demonstrated a high frequency of of spontaneous loss of high molecular weight plasmids coding for virulence in strains of *Shigella* when they were maintained on stock medium for long periods (Sansonetti et al., 1982; Silva et al., 1982; Takabashi et al., 1988).

In this study six of the twenty-one *Shigella* isolates employed in the rabbit illeal loop ligation test to assay for heat labile enterotoxin were found to produce significant fluid accumulation in the ligated intestinal loops of the rabbits. Four of these were

S. flexneri, one S. dysenteriae and S. boydii (Table 6.2 and 6.3; Fig. 6.2 and 6.3). Previous workers have been able to demonstrate fluid accumulation in the ligated iluem of experimental rabbits (Keusch et al., 1972 and Moons et al., 1974). In the case of Shigellae, positive response has been limited to S. dysenteriae and S.flexneri (Keusch et al., 1972) and S. sonnei isolates (Keusch and Jecewicz, 1977). Shigella boydii has not been reported to cause fluid accumulation in ligated illeal loop.

Four of the eight isolates screened for heat stable toxin gave positive response in rabbit illeal loop test. There has been no previous report of heat stable toxin production by any of the Shigella species tested. Keusch et al. (1972a) obtained a negative response when Shigella toxin was heated for 30 min. at 90 C. This result agreed with that obtained by Adetosoye (1981) (Unpublished data) when E. coli enterotoxin filterate heated at 99 C for 30 min. gave negative result in ST assay whereas when these filterates were heated at 65 C for 30 min. fifty-four out of 100 filterates produced positive result (Adetosoye et al., 1984). Thus it could be said that 65 C is the optimum temperature for ST assay.

The histopathological changes observed from ileal sections exposed to LT enterotoxin show that generally there was necrosis, haemorrhage and cellular infilterations (mostly neutrophils) of the lamina. Oedema of the submucosa was observed. This is in agreement with the work of previous investigators including Arms et al. 1965, Levine et. al. (1973) who found similar histological alterations. Keusch et al.(1972a) demonstrated that S. dysenteriae 1 enterotoxin produce alterations and extrusion of villus epithelial cells into the intestinal lumen when inoculated into the ligated illeal loops of rabbits. These workers also found large numbers of transmigrating lymphocytes and considerable nuclei debris scattered throughout the lamina propria. Goblet cell depletion which is a special feature of V. cholera enterotoxin was not encountered with the Shigella isolates employed in this study. Earlier investigators (Steinberg et al., 1975) also failed to demonstrate the depletion of goblet cells.

The mechanism by which diarrhoea is caused by Shigellae has always been a subject of controversy. It is hard to believe that the prominent necrosis and ulceration does not totally reflect an important feature in the overall diarrhoea syndrome (Grady and

Keusch, 1971). The question is whether these changes precede the mucosal demand for more fluid to excrete or are independent and caused by another mechanism. Studies of intestinal perfusion done in monkeys infected with Shigella dysenteriae demonstrated that there were consistent transport abnormalies of salt and water occuring in the colon (Rout et al., 1975). The magnitude of this colonic absorption defects correlates with the degree of bacterial invasion of the small intestine. Fluid is secreted in the jejunum and diarrhoea is manifested when the colon is unable to absorb fluid entering it from the small intestine (Formal et al., 1983).

The absence of bacterial invasion of the colon suggests that enterotoxins may be responsible for the jejunal fluid secretion (Formal et al., 1983). Infact the argument in favour of bacterial invasion as a necessary step in diarrhoea pathogenesis seems to have been mitigated. It has recently been learnt that non-invasive but colonizing enteric pathogens such as enteropathogenic serotypes of E. coli (which do not produce either heat labile or heat stable E. coli toxin) and strains of Vibrio cholerae deleted of genes

for both A and B subunits of cholera toxin, do cause diarrhoea and produce a toxin closely related to Shigella toxin (Levin et al., 1983; O'Brien and La-Veck, 1983).

Toxin binding studies have implicated the presence of developmentally regulated toxin receptors to fluid secretory response (Bresson, 1984; Fuchs et al., 1986). In studies involving neonates and adult rabbit Mobassaleh et al. (1988) provided the first direct evidence of a role for the "Gb " glycolipid-binding site for a functional receptor for Shigella toxin mediating enterotoxin (fluid secrtory) response of the small intestine. Future research will undoubtedly unravel the actual boichemical pathways leading to the production of fluid in the intestine.

It is noteworthy to mention the inability of provoking diarrhoea in laboratory animals fed with Shigella isolates. None of the isolates could be recovered from the faeces of orally infected animals which had been pretreated by starvation and application of calcium carbonate; neither was any febrile condition noticed. A number of reasons have been advanced for the seemingly ineffectiveness of oral inoculation of pathogenic organism is mice and guniea pigs.

These include the antagonism of normal intestinal enteric flora to Shigella (Freter, 1962). There have also been publications on colicin and other antibiotic substances produced by certain E. coli which inhibit the growth of other bacterial species in the gut (Freter, 1956). The action of immune antibodies have also been implicated as a protective mechanism (Adamus et al., 1980).

Starvation, opiates and streptomycin pretreatment have been used singly and in combination to compensate for the natural resistance of animals such as guinea pigs, rats and mice to challenges with Shigellae (Grady and Keusch, 1971). However in this highly manipulated animals, infections often differ from the usual clinical pattern of human disease and knowledge gained from such studies have been subjet of controversy (Keush et al., 1976).

This investigation has conclusively revealed that some Shigella organism are capable of causing diarrhoea through their invasive actions. Some of them also have the capability of elaborating both heat stable and heat labile toxins.

TABLE 6.1

Invasiveness (by Sereny Test) of Shigella isolates from clinical diarrhoea cases

Isolates Number	ISOLATE	EYE	RESULTS AFTER 24hr	48hr	72hr	96hr
1	S. flexneri 3	RIGHT	–	+	+++	+++
2	S. flexneri 1	"	–	–	–	–
3	S. flexneri 6	"	+	++	+++	+++
4	S. flexneri 2	"	–	–	–	–
5	S. dysenteriae 1	LEFT	–	+	++	+++
6	S. boydii	"	–	–	–	–
7	S. boydii	"	–	+	++	+++
8	S. dysenteriae	RIGHT	–	–	–	–
29	S. boydii	"	–	–	–	–
22	S. dysenteriae 6	"	–	–	–	–
33	S. flexneri 2	"	–	–	–	–
36	S. flexneri 2	"	–	–	–	–
37	S. flexneri 4	"	–	–	–	–
44	S. flexneri 6	"	–	–	–	–

KEY: + = Positive Sereny Test i.e. production of *mucopurulent discharge/reddening* of the eye.

– = Negative (See fig. 6.1)

TABLE 6.2

Enterotoxigenicity of *Shigella* isolates using the ileal loop ligation test, (Heat labile enterotoxin)

Isolate No.	Loop No.	Isolate	Lenght of loop (cm)	Volume of fluid	Volume Length ratio	Remark
Control	1	*V. cholera* Og.19339	6.5	7.0	1.07	+ve
"	2	*E. coli* 36004	5.5	2.0	0.3	-ve
2	3	*S. flexneri* 1	7.0	3.5	0.5	-ve
3	4	*S. flexneri* 6	5.0	5.6	1.01	+ve
16	5	*S. flexneri* 1	5.5	4.1	0.7	-ve
29	6	*S. dysenteriae* 2	5.0	4.0	0.8	+ve
20	7	*S. flexneri* 6	6.5	1.5	0.2	-ve
27	8	*S. dysenteriae*	6.0	3.0	0.5	-ve
19	9	*S. flexneri*	5.0	4.0	0.8	+ve
23	10	*S. flexneri*	5.0	2.0	0.4	-ve
Control	11	*E. coli* 36004	7.0	1.5	0.2	-ve
"	12	*V. cholera* 19339	5.0	5.5	1.01	+ve
26	13	*S. dysenteriae*	4.5	1.0	0.22	-ve
24	14	*S. flexneri* 5	5.0	2.0	0.4	-ve

Table 6.2 Contd.)

Isolate No.	Loop No.	Isolate	Lenght of loop (cm)	Volume of fluid (ml)	Volume / Length	Remark
17	15	S. flexneri 2	6.0	2.5	0.4	-ve
30	16	S. boydii	6.0	6.5	1.08	+ve
6	17	S. boydii	5.0	2.5	0.5	-ve
32	18	S. flexneri 2	5.6	2.5	0.44	-ve
44	19	S. flexneri 1	6.0	2.0	0.33	-ve
46	20	S. flexneri 2	6.0	2.0	0.33	-ve
N.T	21	S. flexneri 2	N.T	N.T	N.T	N.T
4	22	S. flexneri 2	6.0	2.5	0.4	-ve
26	23	S. flexneri 2	5.5	1.5	0.24	-ve
Control	24	Classical V. cholera 154	6.0	1.5	0.25	-ve
"	25	V. cholera Ogawa 21140	6.5	2.0	0.31	-ve
33	26	S. flexneri 2	5.5	4.0	0.8	+ve
48	27	S. flexneri 2	3.0	1.0	0.33	-ve
18	28	S. flexneri 4	5.0	2.0	0.4	-ve
45	29	S. flexneri 2	4.5	3.0	0.66	-ve
49	30	S. flexneri 6	4.0	4.0	1	+ve

5	31	S. dysenteriae	5.0	2.0	0.4	-ve
21	32	S. boydii	5.5	2.5	0.4	-ve
34	33	S. flexneri 2	6.0	2.5	0.4	-ve
29	34	S. dysenteriae	5.5	1.5	0.24	-ve
37	35	S. flexneri 4	5.0	1.5	0.24	-ve
38	36	S. dysenteriae	5.5	1.0	0.18	-ve

KEY

N.T = Not tested

+ve = Values greater than 1

-ve = Values less than 1

TABLE 6.3

Enterotoxigenicity of Shigella isolates using the ileal loop - ligation test (Heat stable enterotoxin i.e filterate heated to 65 C for 30min)

Isolate No.	Loop No		Length of loop (cm)	Vol. of fluid (me)	Volume Length	Remark
20	37	S. flexneri	4.0	1.2	0.3	-ve
19	38	S. flexneri	3.5	1.5	0.4	+ve
26	39	S. dysenteriae	5.0	1.5	0.24	-ve
24	40	S. flexneri	5.2	4.0	0.79	+ve
17	41	S. flexneri	5.5	3.5	0.6	+ve
30	42	S. boydii	4.0	3.1	0.75	+ve
44	43	S. flexneri	4.5	1.3	0.3	-ve
27	44	S. dysenteriae	5.0	1.3	0.26	-ve
	C	E. coli 36004	3.5	1.5	0.4	+ve

Fig. 6.1 Keratoconjunctivitis in Guinea pig caused by S. flexneri (isolate 3).

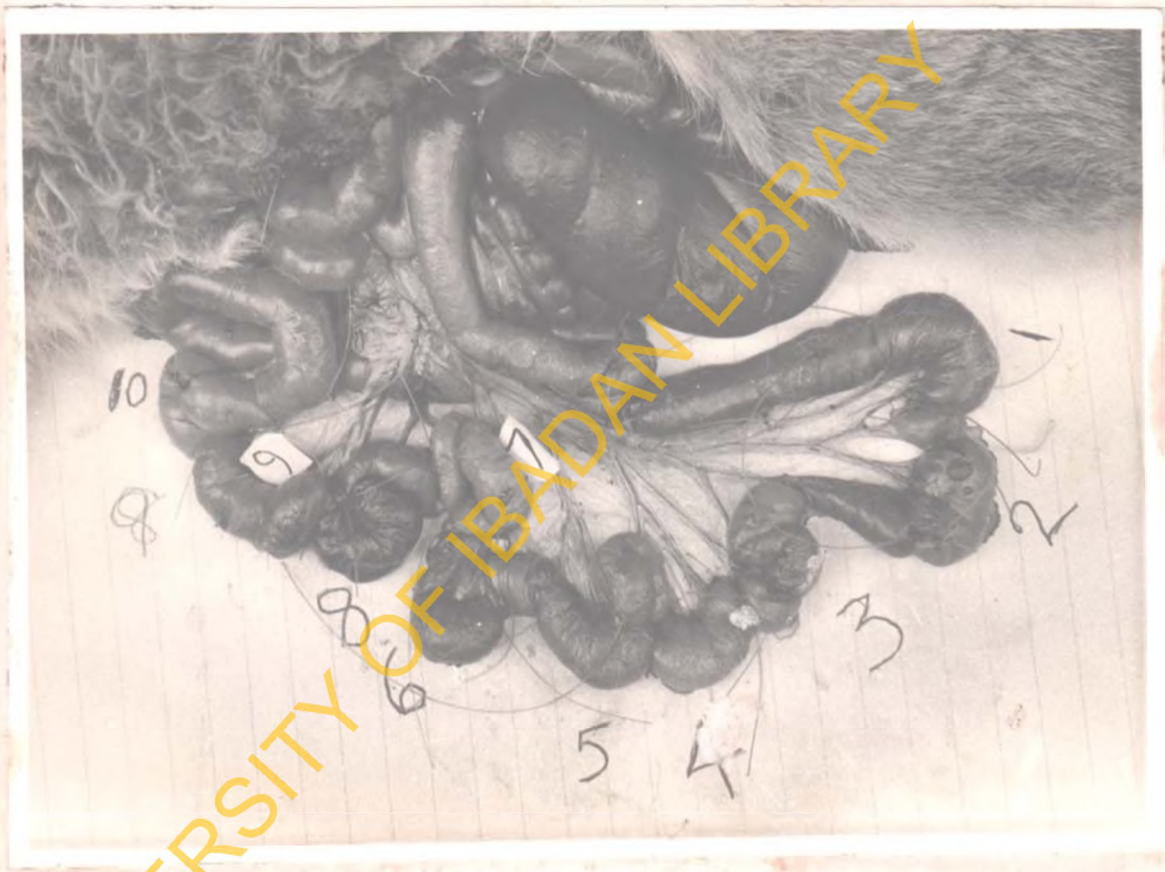


Fig. 6.2 Ileal loop ligationtest. Nos 1-10 show Shigella spp inoculated into each 10cm segment.

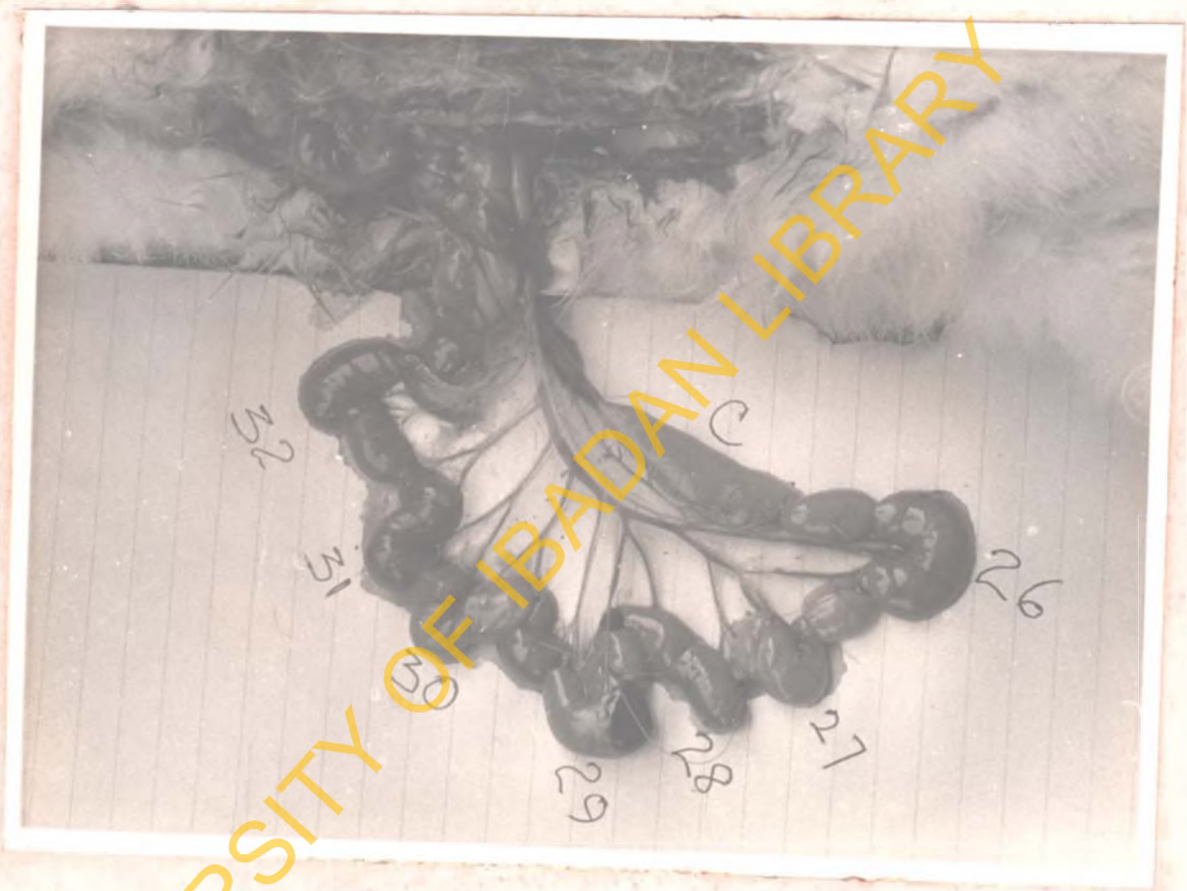


Fig. 6.3: Ileal loop ligation test. C = Control organism (V. cholera Og. 19339). Nos 26-32 shows Shigella spp inoculated into each 10cm segment.

C = V. cholera 26 and 30 are positive S.flexneri, 27, 29 are negative S.flexneri; 31 negative S. dysenteriae and 32 negative S. boydii.

CHAPTER SEVEN

SUMMARY AND CONCLUSION

In this study, stool samples from diarrhoeic patients attending outpatient clinics in three different hospitals in Ibadan were screened for aetiological agents of diarrhoea such as Salmonella and Shigella. The hospitals were: The University College Hospital and States Hospitals at Adeoyo and at Ring Road, Ibadan. Samples were also collected from diarrhoeic piglets at the Teaching and Research Farm, University of Ibadan. The latter were collected using sterile cotton wool swabs.

Selenite F was used as an enrichment medium to enhance the isolation of Salmonella species while potassium tellurite was added to MacConkey agar for the isolation in Shigella. Deoxycholate Citrate Agar (DCA) and MacConkey agar were employed as selective media.

The biochemical characterization of the isolates was performed according to the method described by Edward and Ewing (1962) and Cowan and Steel (1974). The Shigellae were further subdivided into various

serotypes with commercially prepared agglutinating sera. The Salmonellae were also classified into S. typhi and Salmonella spp.

The isolation frequencies of both Shigella and Salmonella were generally low. However, this work established that Shigella organisms are more prevalent than Salmonella in human diarrhoe in this environment. In this investigation 31 Shigella isolates and 22 Salmonella organisms were isolated. The distribution pattern of the serotype showed that S.flexneri was the most frequent (24) followed by S. dysenteriae. (4) and S. boydii (3), S. sonnei was not isolated. One of the Shigellae was isolated from diarrhoeic piglet and was identified as S. flexneri. The later did not show any unusual characteristic from other S. flexneri isolates. The piglet may have become infected through ingestion of feed or water contaminated by Shigella organisms. By comparing the number of different species isolated in this work one can conclude that S. flexneri is more prevalent than other serotypes in this environment.

Among the Salmonella species encountered in this investigation S.typhi predominates on the average as one out of every three Salmonellae isolated. Other Salmonella species apart from typhoid bacilli accounted for roughly two-thirds of the total number. They are all potential agents of diarrhoea in man and involves the animal reservoir. Their mode of transmission may be through food (or water) in which multiplication usually takes place. In this country and other developing countries many lives are lost annually due to typhoid and other Salmonella infections. This can be checked by provision of good drinking water, better hygenic, living conditions and enforcement of public health control measures. The latter include prevention of suspected carriers from handling food stuffs meant for sale to the public. Also effective immunization campaigns will serve to reduce the incidence of typhoid fever.

The antibiotic susceptibility of Shigellae and Salmonellae isolated from diarrhoeic human beings in this work was investiagated. The disc diffusion as well as the Minimal Inhibitory Concentration (broth dilution) procedures were employed. With the disc

diffusion method a total of twenty-one resistance patterns to the eight antimicrobial agents used for the tests were observed. Some of the isolates were found to be resistant to four, five, six and seven drugs. The most common resistant pattern was T-CT-F-A-S-C-Te and it accounted for twenty percent of the total resistant patterns. This resistant pattern was more common among the Shigella isolates. On the other hand pattern CT-F-S and CT-S which occured prominently among the Salmonella isolates were not encountered with the Shigella isolates. It was found that the MIC of ampicillin for Salmonella isolates were generally high as most were inhibited at concentrations between 16 to 128ug/ml. The Shigella organisms were even more resistant as a significant number of them were not inhibited at concentrations higher than 128ug/ml. A larger percentage of the Shigellae were more susceptible to streptomycin at 128ug/ml than chloramphenicol and tetracycline at the same concentration. It was observed that at concentrations lower than 8ug/ml virtually all the four drugs were ineffective.

The practical use of drug resistance survey is to guide clinicians in the choice of drugs to administer in treatment. However, this investigation has shown that most of the antimicrobial agents investigated are not useful as a result of drug misuse. The emergence of multiple drug resistance phenomenon has been blamed on indiscriminate use of antibiotics either as prophylaxis, for treatment of diseases and as well as growth promoter (Anderson, 1968).

Such resistance can either be chromosonally or plasmid mediated (Jacoby and Swartz, 1980). In this investigation, some of the resistances were found to be transmissible and therefore were extrachromosomal.

The ampicillin resistance determinant was transfered in every isolate to E. coli k_{12}. Chloramphenicol, Streptomycin and tetracycline resistance determinants were also transfered along with Ampicillin resistance determinants in some of the isolates. From these results it was shown that some of the resistance determinants were chromosomal since not all determinants were transfered to E. coli K12.

The plasmids coding for these resistance were characterized using gel electrophoresis. They were found to range between 2.2 and 38Mdal. The chloramphenicol R factor was found to be located on the 4.0Mdal. plasmid, ampicillin 13.3, tetracycline 36 and streptomycin 38. The ampicillin resistance plasmid was present in all except one of the transconjugants screened. The triple resistance (A-S-T and A-S-C) determinants were generally found on low molecular weight plasmids (between 2.2 and 5.6Mdal). In addition to their R plasmids most of the isolates carried other plasmids which were transfered to the recipient (Fig. 4.1).

In this work, twenty-one out of thirty-one Shigella and eight out of twenty-two Salmonella isolates were discovered to habour plasmids whose number vary from one to ten per isolate. The Shigella isolates exhibited a large number of small cryptic plasmids and a few large ones. The sizes of the plasmids range between 2.0 and 44.0Mdal. The Salmonella species possess fewer plasmids whose sizes range between 2.0 and 55.5Mdal. This result is in

agreement with the work of Olukoya et al. (1988a) who isolated a significant number of of small cryptic plasmid whose size range between 1-45Mdal from strains of pathogen E. coli, Salmonella spp, Yersinia spp, Shigella spp, Campylobacter spp and Neisseria gonorrhoeae.

The virulence of the Shigella isolates from diarrhoeic cases was investigated in this work. Three different experimental approaches were employed in the investigation. First, oral inoculation of mice and guinea pigs was carried out in order to produce Shigellosis in the animal models. Second, the invassive capabilities of some of the isolates were investigated by inoculating known numbers of the isolates into the cornea of experimental guinea pig models (Sereny test). Lastly, assay for enterotoxin production was carried out using ligated ileal loops of experimental rabbits.

Oral inoculation was unsuccessful as none of the animals came down with Shigellosis. Previous investigation by Freter (1962) failed to produce Shigellosis in guinea pig. The reason advanced for that was the antagonism of normal enteric flora to the inoculated Shigella. However, four out of the

fourteen isolates used in this work produced kerato-conjunctivitis in the guinea pigs used in the experiment. These findings agreed with those of others (Sereny, 1957 and LaBrec, 1964) who produced keratoconjunctivitis in guinea pigs inoculated with Shigella cultures.

Six of the twenty-nine Shigella isolates screened produced heat-labile toxin in the ileal loop of rabbit. The toxin was accompanied by histological changes such as haemorrhage, glandular degeration with moderate lymphocytic infilteration. Furthermore, four isolates produced heat stable enterotoxin as culture filterates of these isolates heated to 65 C for 30 mins. dilated ileal loops of rabbit with the accumulation of fluid. One of the four isolates, identified as S. boydii also produced heat labile enterotoxin.

From these studies it could be concluded that the pathogenicity of Shigella is indeed a two-way process invasion of epithelial cells and toxin production. The latter step has been confirmed by recent discoveries of the presence of toxin receptors which mediated fluid secretory response in neonate and adult rabbits (Mobassaleh et. al., 1988). Future research work

would unravel the actual biochemical pathways leading to the production of fluid in the intestine during diarrhoea episodes.

This investigation has opened new areas of research namely:

(1) Epidemiological use of plasmid profile in differentiating and identifying strains of bacteria involved in enteric infections.

(2) Mode of action of *Shigella* toxins.

(3) Biochemical pathways which occur in the enterotoxin induced by *Shigella* in cases of diarrhoea.

(4) Development of vaccine against *S. flexneri* which is the most common in this environment.

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APPENDIX I

ANTIBIOTICS		CONCENTRATION
Ampicillin	(Amp)	25 mcg
Colistin Sulphate	(CT)	10 "
Nalidixic acid	(Na)	30 "
Nitrofurantion Compound	(F)	200 "
Chloramphenicol	(C)	25 "
Streptomycin	(S)	25 "
Tetracycline	(TE)	50 "
Co-Trimoxazole	(SXT)	25 "

APPENDIX 2.b

Scheme for bulk dilution of antibiotics in the macrobroth or agar dilution used in susceptibility testing (modified from Ericsson and Sherris 1971).

Tube No.	Conc. of antibiotics (ug/ml or u.i/ml) (a)	Volume taken (ml) (b)	Sterile distilled water added (ml) (c)	Total Vol. (ml)	Intermediate conc. (ug/ml i.u/ml)	Final conc. at 1/10 in agar plate
1	2000	12.8	7.2	20	1280	128
2	1280	3.0	3.0	6	640	64
3	1280	2.0	6.0	8	320	32
4	1280	2.0	14.0	16	160	16
5	160	3.0	3.0	6	80	8.0
6	160	2.0	6.0	8	40	4.0
7	160	2.0	14.0	16	20	2.0
8	20	3.0	3.0	6	10	1.0
9	20	2.0	6.0	8	5	0.5
10	20	2.0	14.0	16	2.5	0.25

* For agar plates take 5ml of intermediate conc. add 45ml molten agar cooled to 45-50 C; mix and pour two plates each to obtaim sensitivity agar with indicated final conc.

a = initial stock antibiotic conc. prepared from power or ampoule.

b = Volume of the stock conc. taken

c = Volume of distilled water added to 'b' to obtain total volume.

TABLE 3.1 (APPENDIX 4.)

BIOCHEMICAL CHARACTERISTIC OF ISOLATES

Characteristics	BACTERIAL ISOLATES												
	Sh flexneri 3	Sh flexneri 1	Sh flexneri 6	Sh flexneri 2	Sh dysenteriae	Sh. boydii	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 6	Sh. boydii	Sh flexneri 6
Lab No.	1	2	3	4	5	6	10	16	17	18	19	20	21	22
Gram -ve, Straight rod	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in KCN medium	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate as C source	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydate Utilization:	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gas from glucoce	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid from:														
Glucose	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 3.1 (contd.)

	Sh flexneri 3	Sh flexneri 1	Sh flexneri 6	Sh flexneri 2	Sh dysenteriae	Sh boydii	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 6	Sh. boydii	Sh flexneri 6
	1	2	3	4	5	6	10	16	17	18	19	20	21	22
Arabinose	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	A	A	A	A	-	A	A	A	A	A	A	A	A	A
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	+	+	+	+	+	+	+	+	-	-	-
Voges Proskaur	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 3.1 (Contd.)

	Sh flexneri 3	Sh flexneri 1	Sh flexneri 6	Sh flexneri 2	Sh dysenteriae	Sh. boydii	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 6	Sh. boydii	Sh flexneri 6
	1	2	3	4	5	6	10	16	17	18	19	20	21	23
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dehydrolase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine decarbonxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 3.1 (Contd.)

	Sh flexneri 6	Sh flexneri 3	Sh dysenteriae	Sh dysenteriae	Sh flexneri 2	Sh. dysenteriae	Sh. boydii	Sh flexneri 6	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 4	Sh dysenteriae
	23	24	26	27	28	29	30	31	32	33	34	36	37	38
Gram -ve, Straight rod	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in KCN medium	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate as a C source	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrate utilization														
Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid from:														
Glucose	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 3.1 (Contd.)

	Sh flexneri 6	Sh flexneri 3	Sh dysenteriae	Sh dysenteriae	Sh flexneri 2	Sh dysenteriae	Sh. boydii	Sh flexneri 6	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 2	Sh flexneri 4	Sh dysenteriae
	23	24	26	27	28	29	30	31	32	33	34	36	37	38
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	+	+	+	+	-	-	+	+	+	+	-	+
Voges Praskaur	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urase	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	+	+	+	+	+	+	+	+

TABLE 3.1 (Contd.)

		H_2S	Arginine dehydrolase	Lysine decarboxylase	Ornithine decarboxylase
Sh. flexneri 6	23	-	-	-	-
Sh. flexneri 3	24	-	-	-	-
Sh dysenteriae	26	-	-	-	-
Sh dysenteriae	27	-	-	-	-
Sh. flexneri 2	28	-	-	-	-
Sh. dysenteriae	29	-	-	-	-
Sh. boydii	30	-	-	-	-
Sh. flexneri 6	31	-	-	-	-
Sh flexneri 2	32	-	-	-	-
Sh. flexneri 2	33	-	-	-	-
Sh. flexneri 2	34	-	-	-	-
Sh. flexneri 2	36	-	-	-	-
Sh. flexneri 4	37	-	-	-	-
Sh dysenteriae	38	-	-	-	-

TABLE 3.1 (contd.)

	Sh. flexneri 2	Sh. flexneri 3	Sh. flexneri 4	Sh. flexneri 6	Salmonella Spp	Salmonella Spp	Salmonella Spp	Salmonella Spp	S. typhi	S. typhi	S. typhi	Salmonella Spp	Salmonella Spp
	45	46	48	49	11	12	13	15	50	51	52	53	54
Gram -ve, straight rod	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	-	-	-	-	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	-	-	-	-	-	-	-	-	-
Growth in KCN medium	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate as a C source	-	-	-	-	+	+	+	+	-	-	-	-	-
Carbohydrate utilization:													
Gas from glucose	-	-	-	-	+	+	+	+	-	-	-	+	+
Acid from:													
Glucose	A	A	A	A	AG	AG	AG	AG	A	A	A	AG	AG
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	A	A	A	A	A G	AG	AG	AG	-	-	-	AG	AG
Dulcitol	-	-	-	-	AG	AG	AG	AG	-	-	-	AG	AG
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 3.1 (Contd.)

	Sh. flexneri 2	Sh. flexneri 3	Sh. flexneri 4	Sh. flexneri 6	Salmonella Spp	Salmonella Spp	Salmonella Spp	Salmonella Spp	S. typhi	S. typhi	S. typhi	Salmonella Spp	Salmonella Spp
	45	46	48	49	11	12	13	15	50	51	52	53	54
Lactose	–	–	–	–	–	–	–	–	–	–	–	–	–
Maltose	–	–	–	–	AG	AG	AG	AG	AG	AG	AG	AG	AG
Mannitol	A	A	A	A	AG	AG	AG	AG	AG	AG	AG	AG	AG
Rhamnose	–	–	–	–	AG	AG	AG	–	–	–	–	AG	AG
Salicin	–	–	–	–	–	–	–	–	–	–	–	–	–
Sorbitol	–	–	–	–	AG	AG	AG	AG	–	–	–	AG	AG
Sucrose	–	–	–	–	–	–	–	–	–	–	–	–	–
Trehalose	A	A	A	A	AG	AG	AG	AG	AG	AG	AG	AG	AG
Xylose	–	–	–	–	AG	AG	AG	AG	A	A	A	AG	AG
Indole	–	–	–	–	–	–	–	–	–	–	–	–	–
Urase	–	–	–	–	–	–	–	–	–	–	–	–	–
Methyl red	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	–	–	–	–	–	–	–	–	–	–	–	–	–

TABLE 3.1 (Contd.)

	Sh. flexneri 2	Sh. flexneri 3	Sh. flexneri 4	Sh. flexneri 6	Salmonella Spp	Salmonella Spp	Salmonella Spp	Salmonella Spp	S. typhi	S. typhi	S. typhi	Salmonella Spp	Salmonella Spp
	45	46	48	49	11	12	13	15	50	51	52	53	54
Voges Proskaur	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine dehydrolase	-	-	-	-	+	+	+	+	+	+	+	+	+
Lysine decarboxylase	-	-	-	-	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	-	-	-	-	-	-	-	-	+	+	+	-	-

TABLE 3.1 (Contd.)

	Salmonella spp	S. typhi	Salmonella spp	Salmonella spp	Salmonella spp	S. typhi	S. typhi	Salmonella spp	S. typhi	S. typhi	Salmonella spp	S. typhi	Salmonella spp
	55	56	57	58	59	60	61	62	63	64	65	66	67
Gram -ve, straight rod	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in KCN medium	–	–	+	+	+	–	–	–	–	–	–	–	–
Citrate as a C source	+	–	–	+	+	+	–	+	–	–	+	–	+
Carbohydrate utilization:	+	–	+	+	+	–	–	+	–	–	+	–	+
Gas from glucose	A	A	AG	AG	AG	A	A	AG	A	A	AG	A	AG
Acid from:													
Glucose	A	A	AG	AG	AG	A	A	AG	A	A	AG	A	AG
Adonitol	–	–	–	–	–	–	–	–	–	–	–	–	–
Arabinose	AG	–	AG	AG	AG	–	–	AG	–	–	AG	–	AG
Dulcitol	AG	–	AG	AG	AG	–	–	AG	–	–	AG	–	AG
Inositol	–	–	–	–	–	–	–	–	–	–	–	–	–

TABLE 3.1 (contd.)

	Salmonella spp	S. typhi	Salmonella spp	Salmonella spp	Salmonella spp	S. typhi	S. typhi	Salmonella spp	S. typhi	S. typhi	Salmonella spp	S. typhi	Salmonella spp
	55	56	57	58	59	60	61	62	63	64	65	66	67
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Mannitol	AG	A	AG	AG	AG	A	A	AG	A	A	AG	A	AG
Rhamnose	AG	-	AG	AG	AG	-	-	AG	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	AG	-	AG	AG	AG	-	-	AG	-	-	AG	-	AG
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Xylose	AG	A	AG	AG	AG	A	A	AG	A	A	AG	A	AG
Voges Proskaur	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	AG	-	AG	AG	AG	-	-	AG	-	-	AG	-	AG

TABLE 3.1 (Contd.)

	Salmonella spp	S. typhi	Salmonella spp	Salmonella spp	Salmonella spp	S. typhi	S. typhi	Salmonella spp	S. typhi	S. typhi	Salmonella spp	S. typhi	Salmonella spp
	55	56	57	58	59	60	61	62	63	64	65	66	67
Arginine dehydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	−	+	+	−	−	+	+	−	+	+	−	+	−

TABLE 4.2A APPENDIX 5

RESULTS OF ANTIBIOTIC SENSITIVITY TESTING OF SALMONELLA AND SHIGELLA ISOLATES

	ISOLATE	Co-Trimoxazole SXT 25	Colistin sulphate CT 10	Nalidixic acid Na 30	Nitrofurantion F 200	Streptomycin S 300	Chloramphenicol Chl.	Tetracycline TE 50	Ampicillin AMP 25
o	E. coli 10418	16mm	16mm	16mm	15mm	16mm	16mm	13mm	14mm
.	Sh. flexneri	6 R	6 R	16 S	8 R	6 R	16 S	6 R	6 R
.	" "	6 R	8 R	16 S	6 R	6 R	6 R	6 R	6 R
.	" "	- -	- -	- -	- -	- -	- -	- -	- -
.	" "	11 R	6 R	13 R	6 R	6 R	6 R	6 R	10 R
.	Sh. dysenteriae	9 R	6 R	14 R	6 R	6 R	16 S	6 R	14 R
.	Sh. boydii	16 S	6 R	13 R	6 R	6 R	6 R	6 R	11 R
.	S. flexneri	6 R	16 S	11 R	6 R	6 R	6 R	6 R	14 R
.	S. flexneri	6 R	10 R	13 R	11 R	6 R	18 R	6 R	6 R
.	" "	6 R	12 R	11 R	6 R	6 R	6 R	6 R	6 R
.	" "	6 R	14 R	16 S	21 R	6 R	6 R	6 R	21 R
.	" "	6 R	11 R	18 S	18 S	6 R	18 R	6 R	16 R
.	" "	6 R	11 R	14 R	17 S	6 R	6 R	6 R	17 S
.	S. boydii	16 S	16 R	13 R	9 R	6 R	17 S	8 R	14 S
.	S. flexneri	13 R	6 R	14 R	18 R	6 R	6 R	6 R	6 R
.	" "	6 R	9 R	14 R	10 R	6 R	6 R	9 R	6 R
.	S. dysenteriae	14 R	13 R	14 R	16 R	6 R	6 R	6 R	6 R
.	" "	6 R	6 R	16 S	11 R	6 R	14 R	9 R	6 R
.	S. flexneri	6 R	6 R	13 R	11 R	6 R	6 S	6 R	6 R
.	S. dysenteriae	6 R	6 R	12 R	18 S	6 R	6 R	9 R	6 R
.	S. boydii	16 S	9 R	13 R	10 R	6 R	6 R	11 R	16 S

TABLE 4.2A (Contd.)

	ISOLATE	Co-Trimoxazole SXT 25	Colistin sulphate CT 10	Nalidixic acid Na 30	Nitrofurantion F 200	Streptomycin S 300	Chloramphenicol Chl.	Tetracycline TE 50	Ampicillin AMP 25
	E. coli 10418	16mm	16mm	16mm	15mm	16mm	16mm	13mm	14mm
1.	S. flexneri	6 R	11 R	13 R	16 S	6 R	6 R	6 R	6 R
2.	" "	16 R	13 R	16 S	17 S	6 R	6 R	8 R	21 S
3.	" "	16 R	14 R	18 S	16 S	16 S	16 S	8 R	21 S
4.	" "	16 R	6 R	13 R	9 6	6 R	6 R	13 S	6 R
6.	" "	14 R	13 R	16 S	16 S	6 R	6 R	8 R	18 S
7.	" "	6 R	6 R	13 R	13 R	6 R	6 R	9 R	6 R
8	S.dysenteriae	6 R	6 R	14 R	12 R	6 R	6 R	9 R	6 R
5.	S. flexneri	16 S	11 R	14 R	14 R	6 R	16 R	16 R	18 S
6.	" "	16 S	21 S	21 S	21 S	21 S	16 R	11 R	21 S
8.	" "	18 S	14 R	13 R	10 R	6 R	18 S	18 S	21 S
9.	" "	6 R	11 R	14 R	18 S	6 R	6 R	6 R	6 R
1.	Salmonella spp	6 R	6 R	14 R	6 R	6 R	6 R	6 R	6 R
2.	" "	11 R	6 R	16 S	6 R	6 R	6 R	6 R	6 R
3.	" "	14 R	8 R	16 R	6 R	6 R	9 R	8 R	6 R
5.	" "	6 R	16 S	12 R	6 R	6 R	6 R	8 R	6 R
0.	S. typhi	16 S	14 R	13 R	18 S	6 R	15 S	16 S	10 R
1	" "	16 S	11 R	14 R	10 R	6 R	14 S	16 S	11 R
2.	" "	16 S	16 R	11 S	16 S	13 R	6 R	17 S	14 S
3.	Salmonella spp	18 S	18 S	14 S	14 R	16 R	18 S	16 S	16 S
4.	" "	16 S	11 R	14 R	13 R	6 R	18 S	16 S	16 S
6.	S.typhi	18 S	10 R	16 S	16 S	6 R	6 R	18 S	19 S

	ISOLATE	Co-Trimoxazole SXT 25	Colistin sulphate CT 10	Nalidixic acid Na 30	Nitrofurantion F 200	Streptomycin S 300	Chloramphenicol Chl.	Tetracycline TE 50	Ampicillin AMP 25
		16mm	16mm	16mm	15mm	16mm	16mm	13mm	14mm
57.	Salmonella spp	16 S	10 R	13 R	13 R	6 R	18 S	16 S	13 R
58.	" "	6 R	6 R	11 R	11 R	6 R	20 S	9 R	6 R
59.	" "	21 S	14 R	21 S	21 S	6 R	20 S	6 R	18 S
60.	S. typhi	16 S	14 R	11 R	16 S	6 R	18 S	16 S	14 S
61	" "	16 S	14 R	16 S	18 S	6 R	16 R	16 S	16 S
62.	Salmonella spp	16 S	11 R	13 R	16 S	18 S	18 S	19 S	16 S
63.	S. typhi	6 R	11 R	11 R	16 S	6 R	20 S	16 S	16 S
64.	" "	16 S	11 R	14 R	16 S	6 R	19 S	17 S	16 S
65.	Salmonella spp	6 R	9 R	12 R	13 R	6 R	16 R	14 S	11 R
66.	S. typhi	6 R	9 R	12 R	12 R	6 R	6 R	6 R	6 R
67.	Salmonella spp	16 R	9 R	12 R	11 R	6 R	6 R	6 R	6 R